Exhibit 2

UNITED STATES DISTRICT COURT
SOUTHERN DISTRICT OF NEW YORK

IN RE: Acetaminophen – ASD-ADHD Products Liability Litigation	**22-md-3043 (DLC)**
This Document Relates To: All Actions	

RULE 26 EXPERT REPORT OF BRANDON PEARSON, MS, PhD

The following report is provided pursuant to Federal Rule of Civil Procedure 26. All opinions stated herein are offered to a reasonable degree of scientific certainty. My qualifications are summarized below and detailed in my curriculum vitae, which includes a list of all my publications from the last 10 years and is attached hereto as Exhibit A. The facts and data I have considered in forming my opinions in this case are referenced in this report and on the List of Materials Considered, attached hereto as Exhibit B. I have never previously testified as an expert in trial or at deposition. My hourly rate is $400.

I. QUALIFICATIONS

I am an Assistant Professor of Environmental Health Sciences at Columbia University with expertise in neuroscience, toxicology, and related fields. Among other things, my work specializes in performing preclinical studies to observe the effects of compounds and other factors on neurodevelopment. I have specifically studied the neurodevelopmental effects of prenatal and perinatal exposure to acetaminophen (APAP) through preclinical methods. Based on my expertise and experience, described further below, I am qualified to offer an expert opinion regarding whether the weight of preclinical evidence supports the conclusion that prenatal exposure to APAP in humans can cause neurodevelopmental disorders such as autism spectrum disorder (ASD) and attention-deficit/hyperactivity disorder (ADHD).

I received my Bachelor of Science in Psychology with a minor in Biology at the University of New Mexico in 2005. As an undergraduate, I was employed as a student intern at Los Alamos National Laboratory from 2000 to 2005 where I studied the ecotoxicology of organic and inorganic contaminants. Following my time in New Mexico, I completed a Master of Science degree at Bucknell University, where my work focused on social stress, stress hormones, and behavior in laboratory monkeys. From August 2007 to August 2008, I worked as a behavioral biologist in the Laboratory of Molecular Pathophysiology at the US National Institute of Mental Health in Bethesda, Maryland. There, I performed studies in mouse models of depression and bipolar disorder and investigated novel experimental treatments.

In August 2008, I commenced my PhD studies in the Behavioral Neuroscience program in the Psychology Department at the University of Hawaii. I was mentored by the late Robert Blanchard, PhD, who was a pioneer of rodent models of neuropsychiatric disorders. As a doctoral student, I studied six genetic models of neurodevelopmental disorders. This work extended my expertise in neurochemistry, neuropeptide signaling, rodent models of autism and other neuropsychiatric disorders and resulted in approximately 12 co-authored research papers.

Following my doctoral degree, I completed a postdoctoral fellowship at the University of North Carolina at Chapel Hill in 2015. The fellowship was part of the prestigious Carolina Institute for Developmental Disabilities (CIDD) T32 postdoctoral training program led by Joe Piven, MD, one of the world's top autism experts. As part of the T32 fellowship, I shadowed clinicians assessing autism using the Autism Diagnostic Observation Schedule in the CIDD clinic. I completed my research studies in the Department of Cell Biology and Physiology under the mentorship of Mark Zylka, PhD, focused on experimental pharmacological treatments for Angelman's syndrome, a rare neurodevelopmental syndrome. I also focused on pharmaceutical and chemical toxicity in neurodevelopmental disorders and neurodegeneration. I completed a second postdoctoral position at the German Center for Neurodegenerative Diseases (DZNE) in Bonn, Germany, under the mentorship of Dan Ehninger, MD. My research focused on epigenetic pathways linking aging to neurodevelopment and the neurodevelopmental impact of parental environmental factors such as advanced paternal age.

In 2017, I was recruited by Columbia University to establish an independent research lab focused on neurotoxicology. Neurotoxicology is a branch of toxicology that specifically focuses on understanding how chemicals, drugs, and environmental factors can impact the structure and function of the nervous system. My research lab interrogates the causes of neurological and aging-associated diseases and their mechanisms using innovative approaches including cell culture, animal models (fish and mice), human observational cohorts, and human biospecimens. Extrapolating data from animal studies to human populations for application in medical and public health settings is fundamental to my work. I teach two courses annually: Integration of Science and Practice, a discussion-based public health ethics class, and Computational Toxicology, a course that I designed that is offered every spring. The Computational Toxicology course covers regulation, read-across, novel approach methodologies, predictive toxicology, omics technologies, and advanced and improved approaches to determining health risks of existing and new chemical agents.

I am a co-investigator and lab director of the Columbia Center for Children's Environmental Health (CCCEH), which is one of the leading sites in the world for studies of prenatal exposures and children's neurodevelopmental health outcomes. I am a project investigator for the Columbia-Northern Plains Superfund Research Center where I lead studies on elevated water metal contaminants in systemic toxicity in novel transgenic mouse models and have an NIH grant to study the neurotoxicity of pesticides in a mouse model of Huntington's disease. I am also an Affiliate Member of the Zuckerman Institute and training faculty for Columbia's Neurobiology and Behavior PhD program. My lab also receives generous support from The Taub Institute for Research on Alzheimer's Disease and the Aging Brain at Columbia for our work on environmental factors and neurodegeneration. In five years at Columbia, I have contributed to securing well over $10 million in research funding for science focused on toxic health effects of human chemical exposures.

I have published 40 peer-reviewed research articles that have been cited over 2500 times. Approximately one-third of my published works are on autism and related neurodevelopmental disorders. My work appears in esteemed journals such as *Nature*, *Nature Communications*, *Molecular Psychiatry*, and the *Proceedings of the National Academy of Sciences*. I have given invited lectures and seminars nationally and internationally to departments of genetics, neuroscience, pediatrics, gerontology, and environmental biology. I am currently on the editorial

board for the journal *Toxics* and am an Associate Editor for the journal *Frontiers in Cellular Neuroscience*. I am a member of the Neurotoxicology and Alcohol (NAL) NIH study section where I review grant proposals. I was recently invited to join as technical advisor for a National Institute of Environmental Health Sciences-led systematic scoping review on the association between environmental exposures and ASD. I was previously an ad hoc expert grant reviewer for the French Agence Nationale de la Recherche on a Scientific Evaluation Panel called "Contaminants, ecosystems and health." I am a peer reviewer for numerous articles a year from across 25 journals covering neuroscience, genetics, psychiatry, and environmental health. I am a member of the Society of Neuroscience and a Member of the Society of Toxicology with memberships in the Neurotoxicology Specialty Section and the Reproductive and Developmental Toxicology Specialty Section.

I have studied acetaminophen (APAP) toxicity for approximately 10 years. I first studied APAP neurotoxicity in cell culture and in mice as a postdoctoral fellow at UNC-Chapel Hill. I evaluated the gene expression influences of various doses of APAP and N-acetyl-p-benzoquinone imine (NAPQI) in cultured cortical cells, expression of oxidative stress, and glutathione precursor genes in the livers and brains of pregnant mice and their offspring as well as DNA damage (γ-H2AX and 53BP1) in the brain and gene expression in the brain of offspring dosed acutely with higher doses of APAP, as well as the influences of APAP and NAPQI on mitochondrial superoxide production in cultured primary cortical cells. I also evaluated the influence of APAP and NAPQI on microtubule dynamics in cell free polymerization assays. At Columbia, I led research in mice focused on prenatal and early postnatal APAP exposure and neurobehavioral and transcriptional outcomes, which showed sex-specific communication and activity changes that were accompanied by remarkable and statistically significant changes in forebrain gene expression. This research was part of inter-disciplinary work with Andrea Baccarelli, MD, PhD, MPH and researchers in Canada who led prospective birth cohort analyses of meconium APAP levels and ADHD outcomes and neuroimaging work that was led by a doctoral student that Dr. Baccarelli and I co-mentored (Brennan Baker, now a postdoctoral scientist at the University of Washington). This study was recently published in the journal, *Neurobiology of Disease* (Baker et al., 2023).

I have broad expertise in biology, psychology, neuroscience (molecular, cellular, and systems), neurodevelopment, pharmacology, toxicology, experimental methods, environmental science, human subjects research, and public health. Among these, I have deep expertise in animal models of neurodevelopmental disorders, neurotoxicology, and neurodegeneration. My expertise in neuroscience is central to my work as a toxicologist. My lab uses comprehensive molecular tools and behavioral phenotyping to reveal disturbances in neurological and physiological functioning at doses of exposures that are much lower than those that might elicit more classic features of neurotoxicology such as intoxication, epilepsy, or cellular death. For example, my group regularly applies RNA-sequencing transcriptomics coupled with gene set enrichment analysis (GSEA) and other bioinformatics tools to resolve human health and disease relevance.

II. EXECUTIVE SUMMARY

Utilizing a Weight of Evidence methodology, I analyzed published preclinical studies evaluating the effects of gestational and perinatal APAP exposure on neurodevelopmental disorders, including, among other evidence, studies modeling these effects in animals. Based on this review, I hold the following opinions:

1. Over the last four decades, the overwhelming majority of preclinical studies investigating the effect of APAP on neurodevelopment show that APAP causes neurodevelopmental disruption.

2. Preclinical studies show that APAP is a developmental neurotoxicant capable of causing cascading changes in central nervous system (CNS) structures, molecular pathways, and neurotransmission in offspring exposed to APAP in utero.

3. These preclinical studies show that APAP disrupts neurodevelopment via multiple independent and interacting mechanisms, including oxidative stress, DNA damage, and endocannabinoid system disruption.

4. These preclinical studies strengthen the association seen in epidemiological studies between in utero exposure to APAP neurodevelopmental disorders including ASD and ADHD in humans because the weight of these preclinical studies supports the biological plausibility of that association and because these studies account for confounding factors that may be present in epidemiological studies.

Due to APAP's various effects on the body, it would be impossible to design a single study that would comprehensively capture all the drug's effects. Instead, the evidence of APAP as a neurotoxicant must be viewed in context, as part of a large and well-developed body of scientific work, both epidemiological and experimental. Here, particularly when viewed in its full context, the preclinical data support the conclusion that APAP can cause adverse neurodevelopmental outcomes, including ASD and ADHD, in humans when used during pregnancy in accordance with the dosing information on the product label.

All opinions stated in this report, including the foregoing, are rendered to a reasonable degree of scientific certainty.

III. MANDATE, METHODOLOGY SUMMARY & REPORT STRUCTURE

A. MANDATE

My expert report addresses whether there is a biologically plausible explanation for the increased risk of neurodevelopmental disorders ASD and ADHD in offspring with the prenatal use of APAP and whether the preclinical literature supports that therapeutic clinical and (translationally relevant[1]) preclinical doses of APAP show evidence of harm to the central nervous system, particularly to the developing mammalian brain. This evidence is intended to address the central hypotheses here: (1) Is the proposal of a causal association between in utero exposure to acetaminophen and neurodevelopmental disorders, including ASD and ADHD, consistent with existing biological knowledge and preclinical literature?; and (2) Is there sufficient preclinical

[1] What is "translationally relevant" are rodent doses that are well below those causing acute liver failure and particularly the doses that are analgesic or antipyretic in that species and lower. Routes of administration that are the most reasonable for human exposures (i.e., oral or injection) may receive more emphasis. See Section XI, below.

evidence to conclude that in utero exposure to acetaminophen can cause neurodevelopmental disorders, including ASD and/or ADHD?

This expert report focuses primarily on published experimental (preclinical) evidence evaluating the effects of gestational and perinatal APAP exposure on neurodevelopmental disorders, neurocognitive disruption, behavioral alterations, neurotoxicity, and any other pertinent disruptions to the CNS in animal and relevant cellular models. Cellular models and data from culture systems and computational evidence are included if relevant. Non-CNS outcomes and targets are discussed if they are relevant to indirect CNS toxicity or influence. I did not focus on endocrine disruption or reproductive outcomes except in cases where neurological, neuroendocrine, and neurobehavioral effects were outcome variables.

This expert report does not give an opinion on epidemiological methods, confounding, clinical dose, or human cases unless it relates directly to the preclinical findings discussed. This report discusses neuroscience that is directly relevant to both my work as a toxicologist, as well as the question of whether APAP can cause neurodevelopmental disorders or disruption including ASD and ADHD.

B. Summary of Methodology

Evaluating the causal relationship between a substance and a disease involves gathering, analyzing, and integrating data from various scientific fields (e.g., toxicology, epidemiology, pharmacology). "A single, well-constructed experiment almost never resolves a critical issue on the cause of a disease, especially but not exclusively, diseases resulting from exposure to toxic substances" (Krimsky, 2005). The appropriate methodology for evaluating causation is a Weight of Evidence (WoE) approach—i.e., "a process or method in which all scientific evidence that is relevant to the status of a causal hypothesis is taken into account" (Krimsky, 2005; Organisation for Economic Co-operation and Development (OECD), 2019 ("Weight of Evidence refers to a positive expert opinion that considers available evidence from different independent sources and scientific viewpoints on a particular issue, coming to a considered view of the available, oftentimes conflicting data. It is preferred when every source does not provide sufficient information individually.")). The "ultimate goal" of a WoE evaluation: "to provide a transparent means for communicating decision-making such that decisions can be clearly understood and questioned by all stakeholders" (OECD, 2019; see also Martin et al., 2018 ("The current review [of WoE approaches] also highlighted the value of acquired experience in contributing to expert-informed prescription of the relevant factors to be considered in reporting templates, as a basis for increasing the transparency and defensibility of WOE analysis.")).

This case concerns the causal link between in utero APAP exposure and neurodevelopmental disorders, including ASD and ADHD. Though numerous studies from a variety of scientific fields have yielded information relevant to this inquiry, my investigation and this report focus on toxicology data, specifically developmental neurotoxicology (DNT) data. Toxicology is the study of the effect that chemicals and substances have on people and animals. "Developmental neurotoxicology deals with the study of adverse effects of chemical substances and physical agents on the developing nervous system" (Dubovický et al., 2008). This evidence thus reveals the biological plausibility that exposure to a chemical such as APAP causes neurodevelopmental disorders such as ASD and ADHD.

After reviewing numerous guidance documents, I have drawn from the broadly applicable and systematic approach described in *Guiding Principles and Key Elements for Establishing a Weight of Evidence for Chemical Assessment* (OECD, 2019). Relevant concepts from other guidance documents (e.g., OECD, 2007; Tyl et al., 2008; Center for Drug Evaluation and Research (CDER), 2011) are also incorporated, as appropriate, in my WoE framework. Broadly, the WoE approach that I apply is a systematic review of available data using the series of steps listed below (OECD, 2019), which are described in detail in Section XI, below.

1. Problem formulation: This step involves developing or identifying a hypothesis that will be examined through the review of available data.

2. Evidence collection: This involves establishing lines of evidence and identifying knowledge gaps. For this report, I performed an independent, comprehensive literature search using research databases, including PubMed, to identify relevant literature.

3. Evidence evaluation: This step requires analysis of the lines of evidence to determine data reliability, uncertainty, and relevance. I reviewed all available relevant studies I came across in the process of evaluating the literature, including those that reported an association between prenatal APAP use and ASD and/or ADHD-related outcomes and those that did not find an increased risk in those outcomes.

4. Evidence weighing: This step involves assigning weight to each relevant study. In this report, the weight I assigned to each study translates into a numerical score.

5. Evidence integration and reporting conclusions: This step involves examining the overall significance of the evidence, including coherence among the studies reviewed and the impact of uncertainty.

This approach is consistent with published literature, which recognizes that properly interpreting and integrating DNT data "ultimately requires the exercise of scientific judgment based on an appropriate background, including a knowledge- and experience-based expertise in developmental neurotoxicology" (Tyl et al., 2008). I have applied this methodology with the same rigor as I use in my capacity as a peer reviewer for various scientific journals and as a reviewer for grant proposals for the National Institutes of Health. I rely on my knowledge and experience with conducting and reviewing preclinical studies in this field to evaluate, weigh, and integrate the studies reviewed here and have reviewed and analyzed the studies consistent with how I would review them in my scientific practice and in my capacity as a peer reviewer.

To ensure objectivity and minimize the risk of bias, only objective data from developmental neurotoxicity studies of APAP are included in my WoE assessment. This data has been sorted into the following lines of evidence: in vivo, in vitro/ex utero, and in silico, as described more fully below. But a meaningful WoE assessment cannot be performed in an informational vacuum. Accordingly, I have presented additional facts and analysis, as appropriate, to provide important background information and to ensure a meaningful assessment.

In the event any new, relevant studies or information are published, obtained, or presented following the submission of this report, I reserve the right to review any such data and revise or supplement my report and opinions accordingly.

IV. ACETAMINOPHEN

A. MEDICATION HISTORY

Acetaminophen/paracetamol (APAP) was first synthesized in the late 19th century (Brune et al., 2015; Cendejas-Hernandez et al., 2022). However, it was not marketed for its antipyretic (fever reducing) and antinociceptive (pain reducing) characteristics until the 1950s (Brune et al., 2015). Beginning in 1959, it was available over the counter in the United States (Shaw, 2013). It has subsequently become one of the most widely used drugs worldwide. Since the discovery of APAP's antipyretic and mild analgesic properties, many companies began manufacturing APAP-based products, and it became widely available in many forms, including tablets, capsules, and liquid suspensions.

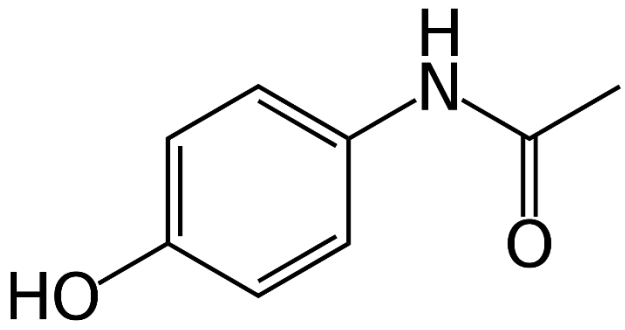


Figure 1 Chemical Structure of APAP

Notably, APAP was not subject to the same rigorous testing process that compounds approved for use in pregnant women are subject to today. [redacted] APAP is used for a wide variety of indications, including headache, muscle ache backache, the common cold, minor pain of arthritis, toothache, muscular aches, cramps, and fever (See, e.g., https://www.tylenol.com/products/tylenol-regular-strength-tablets).

B. APAP FORMULATIONS

In the United States, single-ingredient APAP is sold over the counter ("OTC"), typically for oral administration. The products are available in a variety of formulations and strengths. Importantly, APAP can be hepatotoxic if administered at doses above 4 grams per day in adults (LiverTox, 2016).

C. APAP MECHANISMS OF ACTION AND TOXICITY

APAP is a pleiotropic drug, meaning that its effects are observed throughout the body and involve many parallel pathways and cascading effects. Despite its long and widespread use, APAP's mechanisms of action within the body are still incompletely understood, [redacted],

[redacted]In terms of its therapeutic effects, it appears that the antipyretic and antinociceptive properties of APAP stem from actions on primary and secondary metabolites and their targets including prostaglandin, endocannabinoid, neurotransmitter, and TRPA1 channel signaling systems (Ohashi & Kohno, 2020). Specifically, APAP may inhibit the activity of cyclooxygenases, commonly referred to as COX enzymes, which are a group of enzymes involved in the synthesis of certain prostaglandins. Prostaglandins are chemical mediators involved in pain and fever signaling in the body (Ohashi & Kohno, 2020). Inhibition of COX enzyme activity reduces production of prostaglandins in the CNS, thus reducing pain and fever (Ohashi & Kohno, 2020).

While APAP is an effective antipryetic, its analgesic effect is somewhat limited (Moore et al., 2015). In fact, the common misconception that APAP is harmless, combined with its limited analgesic effect has been posited as a cause of "updosing," and therefore unintentional overdose and injury (Martinez-De la Torre et al., 2020; Wolf et al., 2012). APAP largely does not show peripheral anti-inflammatory properties, suggesting that its pharmaceutical effects occur within the central nervous system or CNS (Ghanem et al., 2016; Mallet et al., 2008). APAP readily crosses the blood-brain barrier and is present in cerebral spinal fluid. It also readily crosses the placenta and enters the fetal brain (Kumpulainen et al., 2007; Levy et al., 1975). APAP has also been shown to produce a calming effect (Stockler et al., 2004), which is consistent with its actions on the endocannabinoid system discussed in more detail later in this report.

When APAP is consumed postnatally, the body begins the process of detoxification through metabolic processes (Ghanem et al., 2016). APAP is detoxified primarily by Phase I and Phase II metabolism (Ghanem et al., 2016). Most ingested APAP, at a therapeutic dose, is conjugated by glucuronidation or sulfation via Phase II reactions, primarily in the liver (Ghanem et al., 2016). This process helps to solubilize APAP and allows the body to process it through renal and biliary clearance (Ghanem et al., 2016).

[redacted]

APAP that is leftover after Phase II metabolism (referred to as "unconjugated" APAP) can be converted into a toxic reactive metabolite NAPQI (N-acetyl-p-benzoquinone imine) by cytochromes P450 (primarily CYP2E1) in the liver, [redacted]. Under normal conditions, NAPQI is detoxified and rendered harmless by conjugation with glutathione (GSH), a powerful antioxidant molecule. However, NAPQI is highly reactive and can cause cellular damage by binding to cellular proteins, leading to oxidative stress and GSH depletion. The process of oxidative stress and its effects on the brain, and specifically neurodevelopment, are described later in this report.

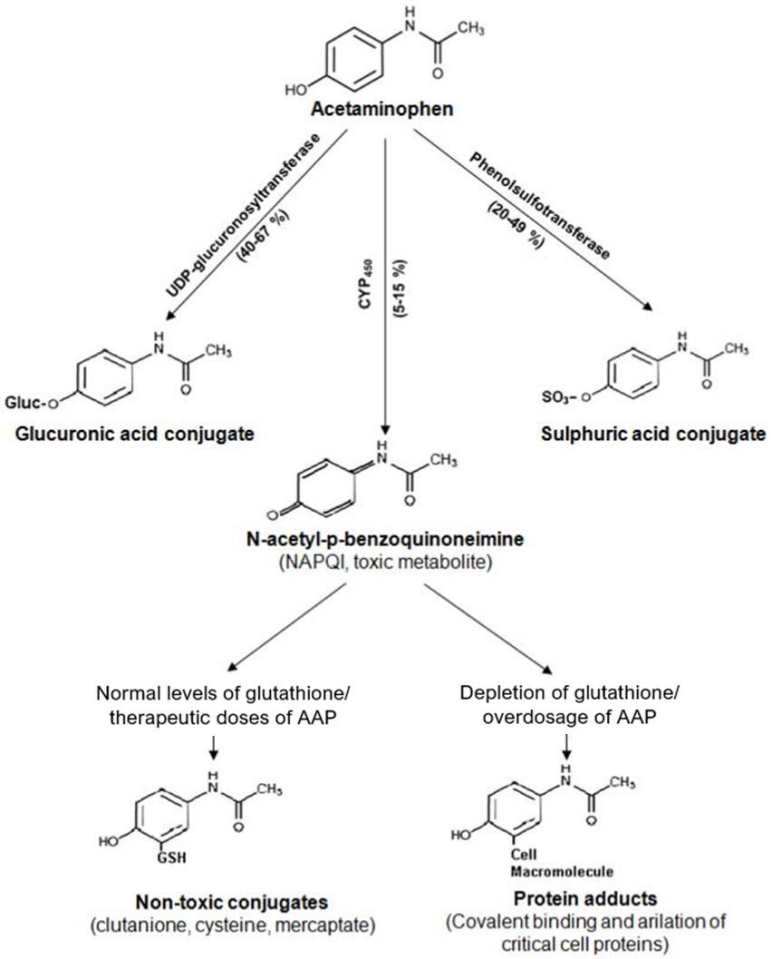


Figure 3. Downstream effects of APAP Metabolism.
Modified from Ghanem et al., 2016.

Unconjugated NAPQI binds covalently with proteins in the body, causing APAP-protein adducts predominantly in the mitochondria (Ghanem et al., 2016). This process causes high levels of oxidative stress, ATP[2] insufficiency, and can cause apoptosis (cell death) which underlies the irreversible tissue damage and liver failure in APAP overdose (Ghanem et al., 2016). The

[2] ATP (adenosine triphosphate) is the molecule that serves as the primary energy currency of cells. It is often referred to as the "molecular unit of currency" because it stores and provides energy for cellular processes.

administration of an antidote called N-acetylcysteine (NAC) may be used to prevent NAPQI-induced liver toxicity. NAC acts by replenishing GSH levels, allowing the body to detoxify NAPQI and preventing further liver damage.

APAP has a narrow therapeutic window, meaning that toxic concentrations are not much higher than therapeutic concentrations (Yan et al., 2018). This sets APAP apart from other over-the-counter analgesics such as ibuprofen or aspirin. Because of APAP's narrow therapeutic window, APAP overdose is the leading cause of acute liver failure in the US, the second leading cause of liver transplantation, and a common contributor to drug overdose hospitalizations (Lee et al., 1996, LiverTox, 2016). [redacted] APAP can cause hepatotoxic effects even at therapeutic doses ([redacted] Harrill et al., 2009). Aminotransferase activity, meaning an elevation of liver enzymes indicative of liver pathology, is seen with therapeutic doses of APAP in both humans (LiverTox, 2016) and rodents (de Mello Miyasaki et al., 2020).

Although APAP has potentially lethal effects in the liver, its effects are not limited to a single organ (Farrell and Miller, 2021). The liver is particularly vulnerable to APAP toxicity given it is the main site of APAP metabolism, but toxic effects occur elsewhere (Farrell and Miller, 2021). Acute liver toxicity represents much of the preclinical and clinical toxicology focus of APAP given the availability of the drug and the short-term, sometimes life-threatening outcomes. In contrast, neurodevelopmental outcomes are more complex, may present much later, are much less fulminant, and can represent broader shared etiologies. Simply put, because APAP is so dangerous to the liver, toxicology research originally centered on APAP's hepatoxicity rather than on its toxic effects on other organ systems. However, renal, reproductive, and nervous system toxicities have also been observed in observational and experimental studies (Kennon-McGill & McGill, 2017). And, as discussed in greater detail below, APAP is a developmental neurotoxicant.

D. Prenatal and Fetal APAP Metabolism

Human and rodent data both demonstrate that APAP readily crosses the placenta and may even accumulate in the placenta and fetal tissues. In human term pregnancy, cord blood and maternal blood APAP levels were essentially equivalent when the mothers were given a single 1 g oral dose prior to scheduled caesarean delivery (Nitsche et al., 2017). In a rat model, researchers anesthetized pregnant rats and placed vascular catheters to sample blood, introduce APAP, and placed microdialysis probes to sample small volumes from fetal tissues including fetal blood to contrast maternal and fetal APAP pharmacokinetics (Chen et al., 2022). The authors found that the levels of APAP were much higher in the maternal circulation after infusion than in the fetal circulation, in contrast to the human study mentioned above. This difference could be attributable to notable differences in placental architecture and permeability between rats and humans (Furukawa et al., 2019).

APAP metabolism in pregnant women and developing fetuses also results in NAPQI formation. In understanding the impact of NAPQI on a fetus, it is important to first note that fetuses and neonates have differential glucoronidation capacity than adults. In other words, a fetus is not simply a smaller adult- their metabolic pathways are fundamentally different. Because of their lower glucoronidation capacity, a fetus is more reliant on sulfation pathways, leaving them with less ability to detoxify NAPQI. This vulnerability to NAPQI means that the pharmacokinetics and

pharmacodynamics that make APAP generally safe for adult use are not present in fetuses. (Andersen et al., 2002; Arana et al., 2001; Ji et al., 2012; Pacifici & Allegaert, 2014; van der Marel et al., 2003). This vulnerability may also mean that NAPQI is present in fetal tissue for longer than it would be present in adult tissue.

Pharmacokinetic analysis of oral APAP administration (single intake 1,000 mg) shows that maternal venous blood (12.3 μg/mL) and fetal blood (11.2 μg/mL) levels are highly correlated (Nitsche et al., 2017). The studies show the formation of NAPQI may increase in pregnancy (Brookhuis et al., 2021). APAP easily passes the placental and blood-brain barrier.

After APAP exposure, NAPQI is also present in the placenta. The placenta is an organ that develops during pregnancy in mammals and serves as the primary interface between the maternal and fetal circulation. The placenta begins to form shortly after fertilization and implantation of an embryo from the chorionic villi of the fetal membrane (small, finger-like projections that extend from one of the fetal membranes surrounding the developing embryo) and the decidua basalis of the maternal uterus (the location where the fertilized embryo implants). The placenta supplies the developing fetus with oxygen and nutrients and removes waste products, including carbon dioxide and urea.

While the placenta generally serves as a protective barrier between maternal and fetal circulations, it is not impermeable to many drugs and other substances. The placental barrier is composed of multiple layers, including the syncytiotrophoblast, cytotrophoblast, and fetal capillary endothelium. These layers work together to restrict the movement of molecules from the maternal circulation to the fetal circulation. However, certain drugs and other substances can cross the placental barrier and expose the developing fetus to potential harm. For example, lipid-soluble compounds, such as opioids, benzodiazepines, and cannabinoids, can easily cross the placenta due to their ability to pass through cell membranes. Water-soluble compounds, such as alcohol and certain antibiotics, can also cross the placenta, but to a lesser extent. However, APAP is demonstrably capable of crossing the placental barrier (Syme et al., 2004).

Animal studies have specifically addressed the differences between adult and pre/perinatal APAP metabolism. Koehn et al., (2019) evaluated the abundance of APAP in the brain relative to plasma in rats following intraperitoneal injection as a function of developmental stage. They compared E19 embryos (exposed via maternal injection) to postnatal day 4 (P4) pups and adults who received direct APAP injections and found that APAP accumulated at higher ratios in brain relative to plasma in the younger rat brains whereas in postnatal periods and into adulthood the ratio becomes relatively equivalent or slightly biased towards peripheral elevations (Figure 4, below). The implication here is that the fetal brain is a sink for APAP due to limitations of fetal brain tissue to metabolize and eliminate APAP in the ways that an adult organism can.

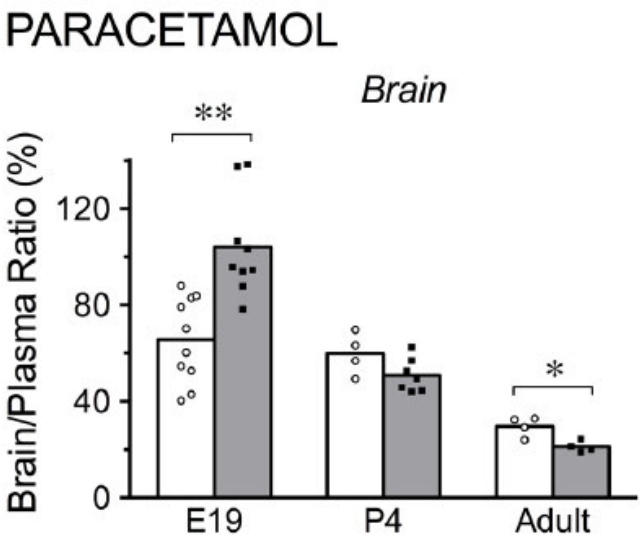


Figure 4. Ratio of brain to plasma radiolabeled APAP in rats as a function of age.
Light bars are acute (single injection) and dark bars are chronic exposures (twice daily injections for four days).
Reproduced from Koehn et al., 2019.

In a later study, Koehn et al., 2021 performed autoradiography (a technique used in molecular biology and research to visualize the distribution and localization of radioactive substances within a sample). Sagittal sections of from P4 and adult radiolabeled APAP-injected mice had brain sections placed on film (the same kind of film that would be used for classic photography). The APAP conjugated to tritium that is present in the brain emits beta radiation that exposes the film and when developed shows the presence and distribution of APAP in the brain of the P4 and adult mice, below. As shown previously by this group, P4 mice (equivalent to 3rd trimester human) accumulate more APAP in their brain than adult mice.

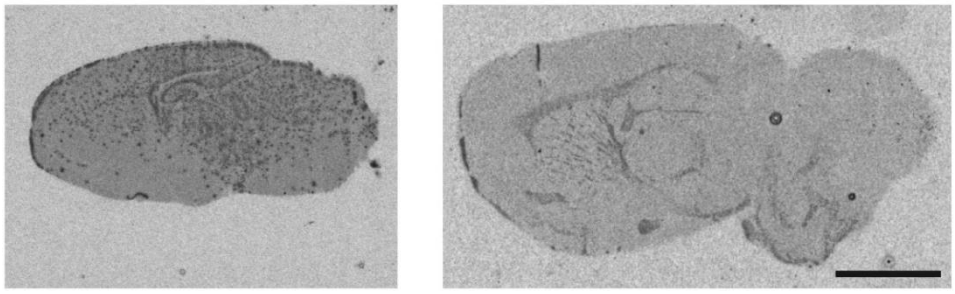


Figure 5. Autoradiography of tritiated (3H-APAP) in P4 (left) and adult (right) mice exposed to the same dose of APAP (15 mg/kg).
Reproduced from Koehn et al., 2021.

E. APAP Absorption, Distribution, Metabolism & Excretion

In human adults, orally administered APAP is 88% bioavailable[3] and is primarily absorbed in the intestines (PubChem, 2023). APAP is "rapidly and uniformly" distributed throughout non-adipose body tissues, allowing for its effects to reach a wide range of areas (PubChem, 2023). Peak plasma concentration occurs approximately 30 to 90 minutes after oral administration and, after 8 hours, only small amounts of the drug are detectible in plasma (PubChem, 2023). APAP can also be deacetylated to 4-aminophenol at the level of the kidney which is capable of causing renal damage (Arzuk et al., 2018).

F. Internal Levels of APAP

A crucial tenet of toxicity is the external and internal dose of a substance. The clinical dose of APAP as well as the concentrations and doses used in preclinical studies are well defined. The internal dose, or the levels that are present or bioavailable in tissues, blood plasma, cerebrospinal fluid and other compartments, is often important in making comparisons across species and interpreting the literature. Accordingly, I have summarized concentrations of APAP in relevant tissues from humans, animal models, and cell culture conditions below.

1. Reported non-toxic blood concentrations of APAP in human blood are 33-132 µM, whereas toxic blood concentrations are reported as 165-992 µM (Buzanska et al., 2009).

2. Human plasma APAP level reached a maximum of 81.4 µM with an IV infusion of 1 g APAP, whereas 39.3 µM was detected in the cerebrospinal fluid from the same dosage condition (Singla et al., 2012).

3. A controlled study comparing healthy participants compared plasma concentrations of APAP in response to IV administration vs oral administration of 1 g APAP. Maximum concentrations (C_{max}) were 86 µM with oral dosing and 125.7 µM with IV administration (van der Westhuizen et al., 2011).

4. Reported mouse serum concentrations of APAP reached a maximum of 774 µM with an oral dose of 150 mg/kg (Saad et al., 2016).

5. Reported rat plasma levels of APAP reached 1000 and 2000 µM for intraperitoneal injections of 250 and 500 mg/kg, respectively. Levels in the cerebrospinal fluid peaked at similar or a bit higher level of 1000 and 3000 µM, respectively (Posadas et al., 2010).

The levels of APAP found in living organisms, and the toxicity as related to dose, are further discussed later in this report.

---

[3] Bioavailability refers to the extent and rate at which a substance is absorbed and becomes available to the body's circulation and target tissues or organs. It is a measure of the fraction of an administered dose of a substance that reaches the systemic circulation in an unchanged form or as an active metabolite.

G. INTERINDIVIDUAL VARIABILITY IN VULNERABILITY TO APAP

Susceptibility to APAP-induced toxicity displays significant interindividual variability, influenced by genetic and non-genetic factors. Genetic variations in drug-metabolizing enzymes, such as CYP2E1 and UGT, can affect APAP metabolism and disposition, and contribute to differences in susceptibility to APAP toxicity between individuals (Krasniak et al., 2014). Common polymorphisms in immune genes such as CD44 underlie variable susceptibility to APAP hepatotoxicity in humans and mouse models (Harrill et al., 2009). Non-genetic factors, such as age, sex, body weight, and the presence of underlying hepatic or metabolic disorders, can also influence the risk of APAP toxicity (Heldring, 2014). Co-administration of drugs that induce or alter the activity of CYP 450 enzymes can also be a risk for increased APAP toxicity (Deodhar et al., 2020).

Elderly individuals with reduced renal or hepatic function may be more susceptible to APAP toxicity due to impaired drug elimination and metabolism (Klotz, 2009), while patients with underlying hepatic or metabolic disorders, such as chronic alcoholism or viral hepatitis, may have decreased tolerance to APAP toxicity (Ghanem et al., 2016). Concurrent exposures to other chemicals that increase oxidative stress can also make one less able to efficiently detoxify and eliminate APAP because of reduced GSH.

Dietary status may likewise affect APAP toxicity. Fasting or malnutrition is considered a risk factor for APAP-induced hepatotoxicity because fasting depletes glycogen stores (LiverTox, 2016). As a result, APAP's half-life increases with decreased glucuronidation and more NAPQI forms, increasing the potential for hepatotoxicity. Fasting also lowers hepatic GSH levels, allowing NAPQI to cause oxidative stress, discussed in more detail below.

Most epidemiological and preclinical studies are not able to fully account for interindividual differences in APAP toxicity. Epidemiologists must either use large sample sizes to see true signals or perform nested analyses or sensitivity analyses to attempt to resolve the best approximations of effects of APAP. Experimentalists on the preclinical side utilize animal and cellular systems that are essentially prototypes. While these systems also have limited ability to account for inter-individual differences, there is innovation in considering what is the true human-relevant background of cumulative experiences to model for added APAP neurodevelopmental influences (e.g., Lichtensteiger et al., 2015).

H. APAP USE DURING PREGNANCY

Despite the fact that it was never subject to the rigorous preclinical testing that other compounds typically require and undergo prior to approval, APAP is currently the only pain medicine and fever reducer that is deemed indicated for use during pregnancy. Antinociceptive doses of aspirin, and antinociceptive or antipyretic doses of ibuprofen are typically deemed unsafe (in ibuprofen's case, during certain periods of pregnancy) because of the risk of complications, although low dose aspirin can be recommended to manage cardiovascular and preeclampsia risks. Other more potent pain medications like opioids are likewise dangerous to embryos or the fetus and are contraindicated. Accordingly, APAP is, in effect, currently the only recommended means to reduce fever and pain throughout pregnancy.

As a result, most studies estimate that more than 60% of pregnant women consume APAP during pregnancy (Bandoli et al., 2020). Studies have found that pregnant women take APAP for a broad spectrum of indications including headache, migraine, general pain, sleep, cramps, and cold or flu (Bandoli et al., 2020). Nearly 20% of pregnant women who reported consuming APAP during pregnancy reported taking it for at least 20 days (Bandoli et al., 2020).

However, APAP's safety in pregnancy is far from settled. [redacted]. Further, Ofirmev, an IV formulation of pure APAP, is a Pregnancy Category C drug (indicating animal reproduction studies have shown adverse effect on the fetus), and the label indicates it should be used "only if clearly needed." The Ofirmev label also notes studies in pregnant rats receiving oral acetaminophen showed evidence of fetotoxicity. Likewise, in Europe, pregnant patients are specifically advised that, "[I]f clinically needed, paracetamol can be used during pregnancy however it should be used at the lowest effective dose for the shortest possible time and at the lowest possible frequency."

[redacted]

[redacted]

Further complicating this issue is the fact that Janssen, the pharmaceutical wing of Johnson & Johnson, has a combination product known as Ultracet (a combination APAP product). In the section of Ultracet's label specifically discussing APAP, it states: "acetaminophen approximately 1.3 times the maximum human daily dose (MRHD) showed evidence of fetotoxicity and increases in bone variations in the fetuses (Ultracet Label). [redacted]

V. HUMAN NEURODEVELOPMENT

A. HUMAN BRAIN DEVELOPMENT

The human brain is the most complicated organic structure on Earth. It is comprised of billions of cells and trillions of connections (synapses). Every thought, sensation, memory, emotion, movement, idea, dream, or physical feat is attributable to the brain. As might be expected, the sophistication of the brain is reflected in the biological processes which govern the brain's prenatal development.

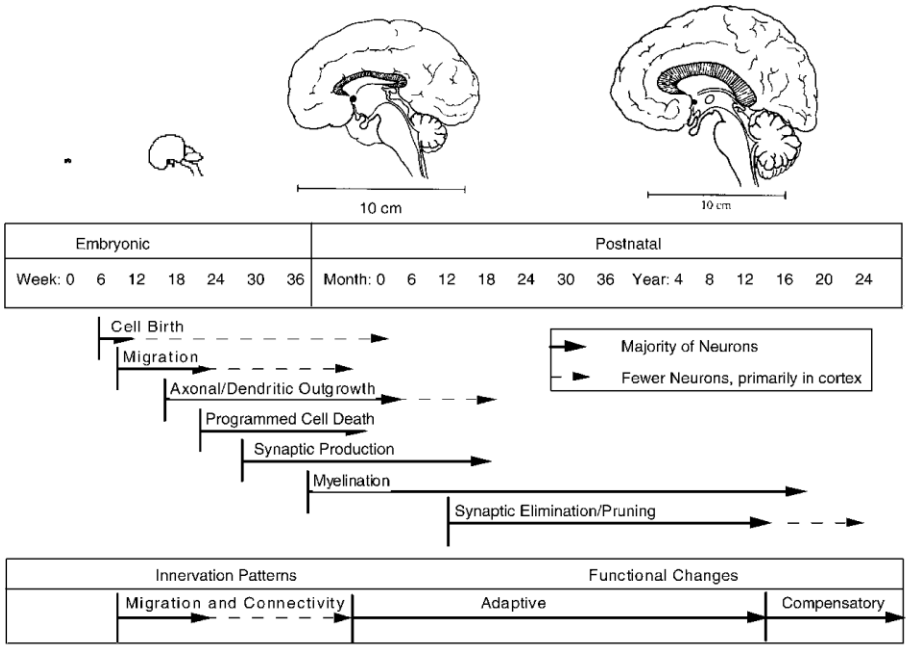


Figure 6. Stages of human brain development.
Reproduced from Andersen, 2003.

Starting with the division of a single-celled zygote after fertilization, a dynamic, adaptive, and rigorous set of processes commence which produce the neural tube by week three, the basic structure of the brain by week nine, and the rapid development of the cerebral cortex and hippocampus in the second and third trimesters. Over the course of the gestational period, cells undergo division, migration, differentiation, connection (synapse formation), and pruning (death). Axons are strengthened through sensation and experience and then myelinated (enclosed in a myelin sheath). By the time of birth, a human baby is born with virtually all the neurons they will ever have. During this period of tremendous gestational growth, brain development is shaped by gene expression, the orientation and formation of neural pathways, and the exposure to gradients of hormone, peptide, and neurotransmitter signals, and to damage or disruption from genetic or epigenetic lesions, environmental toxins, oxidative stress, pathogens, or immune/inflammatory signals.

In utero brain development begins shortly after fertilization and continues throughout pregnancy (Konkel, 2018). In utero brain development is very dynamic and the brain changes every day. Shortly after fertilization, the single-celled zygote divides, eventually forming a blastocyst. The blastocyst then implants into the uterine lining. Once implanted, the cells forming the blastocyst begin to differentiate into the three primary germ layers: the ectoderm, mesoderm, and endoderm. The ectoderm, which forms the outermost layer, gives rise to the neural tube. These early processes of brain development occur within a physically tiny embryo. Necessarily, precise, and sophisticated signals are required from each cell in order that they be correctly differentiated into their final form.

In the third week of embryonic development, the neural tube forms, and the brain as we know it starts to develop (Ackerman, 1992). Approximately 3 mm long, the neural tube begins as a hollow cylinder. Eventually, the hollow space forms the ventricular system of the brain (Stiles & Jernigan, 2010). Over the course of pregnancy, the neural tube goes through a complex process of growth, folding, and differentiation to form various regions of the brain and spinal cord. By week five of embryonic development, three distinct regions (the forebrain, midbrain, and hindbrain) have formed.

Week nine of gestation marks the beginning of the fetal period, as the brain appears as a small structure within the developing fetus. As neuron production, migration, and differentiation begin, the structure of the brain takes on the typical mature pattern of gyral and sulcal folding. By the fourteenth week of gestation, the fetal brain has formed many of its primary structures, including the cerebellum, cerebral cortex, and brainstem.

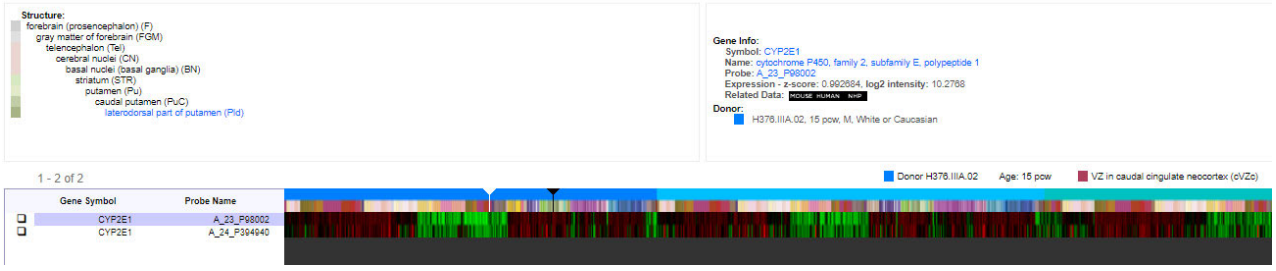
Throughout the second and third trimesters, the cerebral cortex (the outer layer of the brain that is responsible for higher-level thinking and perception) undergoes significant growth. The number of brain cells in the cerebral cortex grows rapidly, and complex folding and differentiation occur, supported by new synapses and neural networks. During the third trimester, the hippocampus, which plays a crucial role in memory and learning, also goes through a period of rapid development. During the third trimester, the brain begins to control basic bodily functions such as breathing and temperature regulation, which will be necessary for life outside the womb. The fetus also begins to recognize and respond to external stimuli such as light, sound, and touch. In time, the fetal brain develops the ability to process and interpret sensory information, perceiving and differentiating between different tastes and smells.

The end of gestation involves programmed cell death, a process of "pruning" neurons and synapses that aids in establishing the networks within the developing brain (Stiles & Jernigan, 2010). The neurons that remain at birth represent the summation of most forms of neurogenesis: they are the lion's share of the neurons a human will ever have. So, while the human brain continues to mature postnatally, the brain is relatively developed at birth. The rigorous and precarious process of in-utero brain development has unambiguous, lifelong implications.

During brain development, CYP2E1 is expressed at various times in various locations in the brain (García-Suástegui et al., 2017). CYP2E1's presence and levels are heterogeneous and show individual variability (García-Suástegui et al., 2017). As mentioned previously in this report, CYP2E1, short for Cytochrome P450 2E1, is an enzyme involved in the metabolism of APAP and the creation of its toxic metabolite, NAPQI. While primarily present in the liver, CYP2E1 is present in other tissues, including the brain. CPY2E1 is also present in the fetal brain in a variety of locations and at different times during fetal development.

Below is a screen shot from BrainSpan, an "Atlas of the Developing Human Brain," which shows the heterogeneity of CYP2E1 expression in different areas of the brain from various mid-gestation brain regions (Allen Institute, 2019, available at https://www.brainspan.org/lcm/search?exact_match=false&search_term=%22CYP2E1%22&search_type=gene).

In the screenshot below (see also Appendix A), note that green shows decreased expression of CYP2E1 whereas red shows increasing expression of CYP2E1. Data are normalized to z-scores, a statistical technique which allows comparison and integration across samples and regions. Various cortical, striatal, and basal ganglia regions show higher z-scores of CYP2E1 enrichment including the bed nucleus of the stria terminalis (BNST) which are areas that, if damaged, could lead to neurodevelopmental disorders such as ASD and ADHD given its central role in neuroendocrine, emotional, and neurobehavioral physiology (Hammack et al., 2021).



B. VULNERABILITY OF DEVELOPING BRAIN

The developing brain is more vulnerable than the adult brain to toxicants. The developing brain's vulnerability (in both humans and animals) stems from several factors, including the immaturity of the developing brain's blood-brain barrier and the rapid degree of cellular differentiation (Lanphear, 2015). The developing brain's rapid cellular differentiation and diversification means that an injury to a progenitor cell would impact all of the neurons and supporting cells that would have been in the lineage of that progenitor.

In addition, neurons need to set up connections and undergo activity dependent maturation and network formation independent of their genetics and epigenetics. Connections that are formed that are not needed need to be pruned and eliminated. Such processes require massive amounts of energy in the form of glucose, oxygen, amino acids, proteins, etc. Cascades of signaling molecules and hormones need to coordinate these various processes and they are all vulnerable to perturbations from outside stressors. Even the most minor and temporary alterations in oxidative stress during these massive waves of intricate brain development can change the course of brain development and create conditions for disordered behavioral function.

In addition, during brain development, an excessive number of cells are initially generated, surpassing what is actually required for proper neural function. Accordingly, the brain undergoes a carefully orchestrated process of programmed cell death to ensure that the optimal number of neurons remain in order to create and maintain connections in the brain.

Developmental neurotoxicity can result in the dysregulation of processes, including neurogenesis, neuronal differentiation, synaptogenesis, and the establishment of functional connectivity networks. A wide variety of molecular or cellular events which lead to developmental neurotoxicity, also known as "adverse outcome pathways," have been identified and analyzed by scientists. During the prenatal development of the brain, a wide variety of critical, parallel, and cascading processes are critical to healthy development. As exposure to neurotoxic chemicals during development can result in developmental neurotoxicity, exposure to chemicals is a major factor in the prevalence of neurodevelopmental disorders (Nishimura et al., 2021).

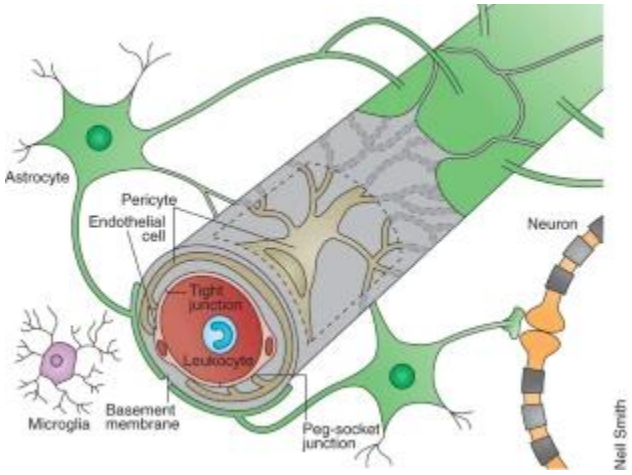


Figure 7. Graphic depiction of cellular interplay at the neurovascular unit, including the blood brain barrier. Reproduced from Obermeier et al., 2013.

Another factor impacting the vulnerability of the developing brain is the immature blood-brain barrier. During early stages of brain development, the blood-brain barrier (BBB) has yet to be fully formed and is considered to be “leaky” which makes the developing brain more vulnerable to drugs and toxins. The BBB is a specialized structure that restricts the passage of substances from the blood into the brain. The absence of a fully functional BBB during neurodevelopment arises from the dynamic and intricate process of blood vessel formation and maturation within the brain. As the brain develops, blood vessels begin to form and grow, initially lacking the complete complement of specialized cells and tight junction proteins that comprise the mature BBB. These

cells and proteins, initially missing or incomplete, go on to play a crucial role in maintaining the integrity of the BBB and regulating the transport of molecules between the blood and the brain. Chemicals, molecules, and other signals from the maternal circulation, including APAP, readily access the developing fetal brain.

C. CONDITIONS IN MOTHER THAT CAN AFFECT FETUS

Multiple conditions in the mother can affect the neurodevelopment of her children, including maternal nutrition, infection, stress and inflammatory processes (Fitzgerald et al., 2020). Certain maternal infections may affect neurodevelopment in children, but timing is important, and the type of effect may depend on the type of infection (Fitzgerald et al., 2020). Similarly, infections can induce inflammation, and those inflammatory processes in the mother can also impact neurodevelopment and leave the fetus at increased risk for other insults (Fitzgerald et al., 2020). When the mother experiences inflammation, immune cells and signaling molecules are activated, leading to the production and release of pro-inflammatory cytokines and other inflammatory mediators. These molecules can cross the placenta and reach the fetal brain, where they can trigger a localized immune response and induce neuroinflammation. Neuroinflammation can activate microglial cells, the resident immune-like cells of the central nervous system, leading to their activation and release of additional inflammatory mediators. This inflammatory response can disrupt the delicate balance of neuronal development and maturation in a developing fetus. Neuroinflammation also has a complex, bidirectional relationship with oxidative stress (which can be induced by APAP use) that is discussed later in this report. The endocannabinoid system and prostaglandins, also discussed later in this report, similarly have a close and complex relationship to inflammation. Accordingly, while many variables in terms of maternal condition can impact the neurodevelopment of the fetus, several mechanisms of action of APAP can actually render a fetus more vulnerable to these effects.

Studies also show that APAP can have a pro-inflammatory effect on the developing brain. While APAP's neurotoxicity is most severe during prenatal and neonatal exposure in animals, APAP can cause damage to the nervous system even in older animals, which further confirms APAP's mechanism of action. Pinto and colleagues (2020) demonstrated that while APAP is a therapeutic intervention for a Down's Syndrome model mouse, it also showed pro-inflammatory effects in wild-type (WT) mice treated with APAP. Namely, three once daily 100 mg/kg injections of APAP were given to young mice and dendritic spines were reduced (Panels E and F left panels in WT mice, indicating alterations to the number and quality of synapses or connections between neurons) as well as cognitive impairments as shown in panels G and H below. What is striking here besides the obvious findings is that low dosage, well below what might cause liver damage, leads to neuroinflammation, synaptic changes, and cognitive changes in mice.

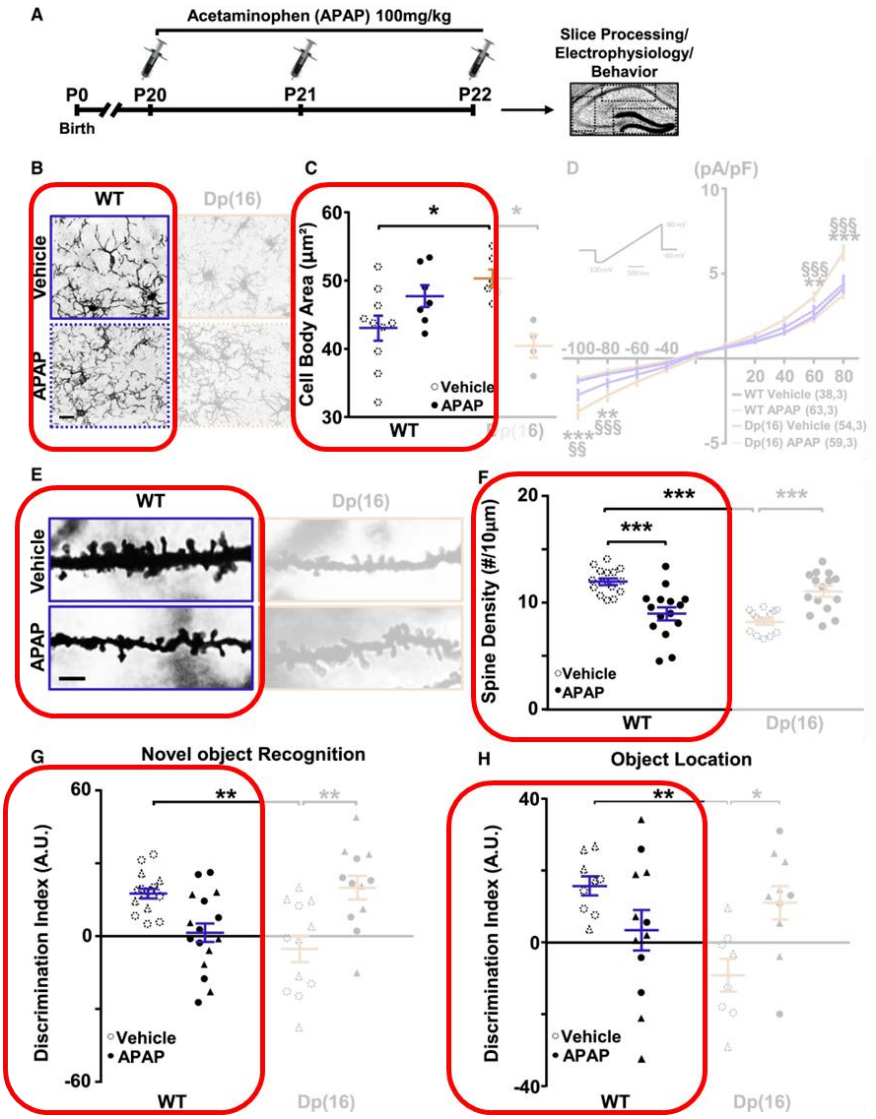


Figure 8. APAPTreatment Rescues Microglial Morphology and Activity, Dendritic Spine Deficits, and Cognitive Impairment in Dp(16) Mice. (A) Experimental protocol. (B) Iba1-stained hippocampal slices from WT and Dp(16) animals treated with either vehicle or APAP. Scale bar: 10 µm. (C) Quantification of the cell body area of microglial cells. Bars represent the average of microglial cell body areas in all analyzed animals ± SEM, and circles represent single data points of the cell averages for each animal (14–73 cells/animal; 1 slice per animal). ⋆p < 0.05; two-way ANOVA; FInteraction (1, 23) = 14.88; p = 0.0008; Holm-Sidak post hoc test. Dotted circles represent data are from C for comparison. (D) Voltage-activated current profile of microglia in WTCX3CR1-GFP and Dp(16)CX3CR1-GFP acute hippocampal slices treated with vehicle or APAP in vivo. Voltage currents were analyzed using ramps ranging from −100 to 80 mV (inset). Data are expressed as average current (picoAmpere,pA) normalized for the capacitance (picoFarad,pF) for each mV step of all analyzed cells ± SEM. ⋆represents the statistical difference between Dp(16) and WT vehicle; ⋆⋆p < 0.01; ⋆⋆⋆p < 0.001; § represents the statistical difference between Dp(16) vehicle and Dp(16) APAP; §§p < 0.01; §§§p < 0.001. Two-way RM ANOVA; FInteraction (27, 1,890) = 9.261; p < 0.001; Holm-Sidak post hoc test. In parenthesis: analyzed cells, animals. The vehicle-treated data are from Figure 1I for comparison. (E) Dendritic spines from Golgi-Cox-stained slices of hippocampi from P22 WT and Dp(16) mice treated with vehicle or APAP. Scale bar: 4 µm. (F) Quantification of the spine density. Bars represent the average spine density of all analyzed cells ± SEM, and circles represent single data points for each cell (3 animals per condition). ⋆⋆⋆p < 0.001; two-way ANOVA; FInteraction (1, 56) = 42.99; p < 0.001; Holm-Sidak post hoc test. The vehicle-treated cell data (dotted circles) are from Figure 3C for comparison. (G and H) Quantification of the discrimination index in the NOR and OLT tests in P22 WT and Dp(16)

mice following vehicle or APAP treatment. Bars represent the average discrimination index of all analyzed animals ± SEM, and symbols (circles, males; triangles, females) represent single data points for each animal. ⁎p < 0.05; ⁎⁎p < 0.01; (G) two-way ANOVA, FInteraction (1, 51) = 23.76, p < 0.001; Holm-Sidak post hoc test; (H) two-way ANOVA, FInteraction (1, 35) = 11.24, p < 0.002; Holm-Sidak post hoc test. The vehicle-treated data (dotted circles and triangles) are from Figures 2E or 2F for comparison.
Modified from Pinto et al., 2020.

VI. NEURODEVELOPMENTAL DISORDERS

Neurodevelopmental disorders (NDDs) are a spectrum of conditions caused by abnormal development in the brain. The DSM-5 defines a neurodevelopmental disorder as a condition that begins in childhood and is characterized by impairments in cognition, communication, motor skills, self-regulation, or social-emotional functioning. Under the current DSM-V criteria, for a condition to be considered an NDD, it must cause impairments that are present in the early developmental period even if not diagnosed until later in life and must cause significant functional impairment in one of more critical areas (for example, interpersonal relationships, work, or school).

For decades, there has been a push to recognize NDDs as broader endophenotypes (heritable traits derived from, here, neurocognitive performance deficits) beyond the traditional boundaries of the DSM-V (Morris-Rosendahl & Crocq, 2020). The NDDs described by the DSM-V are fundamentally a collection of specific behavioral attributes which may have varying etiologies and genetic and environmental causes. In humans, the need for the development of new treatments for ASD and ADHD necessitates the DSM's categories for these behaviors, which are defined below (Thapar et al., 2017). However, as a preliminary step, we must acknowledge that these clinical constructs do not necessarily reflect the shared etiologies that result in these phenotypes (observable characteristics in an individual resulting from the expression of genes).

A. AUTISM SPECTRUM DISORDER

The Diagnostic and Statistical Manual of Mental Disorders, 5th edition (DSM-5)[4] defines autism spectrum disorder (ASD) as a neurodevelopmental disorder characterized by persistent deficits in social communication and social interaction across multiple contexts, as well as restricted, repetitive patterns of behavior, interests, or activities. The term "spectrum" reflects the wide range of symptoms and severity of the disorder, which varies greatly among individuals.

The DSM-5 identifies two core domains of impairment that must be present for a diagnosis of ASD: social communication and interaction, and restricted, repetitive patterns of behavior, interests, or activities. Under the social communication and interaction domain, the DSM-5 specifies that individuals with ASD must have deficits in social-emotional reciprocity, nonverbal communication, and developing, maintaining, and understanding relationships. Under the restricted, repetitive patterns of behavior, interests, or activities domain, the DSM-5 specifies that individuals with ASD must display at least two of four different types of behavior: repetitive

[4] The DSM-5 made several changes to the prior version of the DSM, the DSM-IV, as it relates to ASD and ADHD, which are further detailed in Dr. Hollander's report.

speech or movements, rigid adherence to routines or rituals, fixated interests that are abnormal in intensity or focus, and hyper- or hypo-reactivity to sensory input.

The DSM-5 also includes severity levels for ASD based on the amount of support an individual requires, ranging from level 1 (requiring support) to level 3 (requiring very substantial support). Additionally, the DSM-5 recognizes that some individuals who do not fully meet the diagnostic criteria for ASD may still display significant social communication and interaction deficits or restricted, repetitive patterns of behavior, and provides a diagnosis of social communication disorder (SCD) for these individuals.

Research has already linked in utero exposure to several compounds with autism in children. For example, Valproic Acid (VPA) is an anti-convulsant medication used primarily to treat epilepsy. However, large cohort studies have found a clear link between exposure to VPA in utero and autism in children, even adjusting for maternal epilepsy (Harden, 2013). In animal models, VPA has similarly been shown to induce autism-like behaviors in rats and mice, including social behavior deficits, increased repetitive behaviors, and deficits in communication. In brief, environmental exposures can and often do act as a major cause of ASD, particularly when those exposures occur when the brain is at its most vulnerable (in utero) (Jeon et al., 2018).

In addition, genetic research related to ASD has allowed researchers to identify hundreds of genes that potentially affect the risk for an individual to develop ASD, including over 100 high-confidence genes (e.g., https://gene.sfari.org/). Knowledge of these genes is not only critically important for medical researchers, it also provides valuable information for scientists studying neurodevelopmental disorders in animal models, as further discussed below.

1. Structural and Functional Brain Abnormalities in ASD

Meta- and mega-analyses of ASD brain imaging compared to neurotypical controls frequently show grey matter volume reductions in striatum and amygdala/hippocampus (Lukito et al., 2020). ENIGMA mega-analysis has shown increased cortical thickness in frontal areas and decreased cortical thickness in temporal areas, and a voxel-based morphometry meta-analysis shows cortical abnormalities (e.g., grey matter volume reductions in medial prefrontal cortex and posterior insula) and increased left anterior temporal, right inferior temporo-parietal, left dorsolateral prefrontal and precentral cortices (Lukito et al., 2020).

Lukito et al. (2020) performed a meta-analysis of structural and functional differences in ASD and ADHD brains compared to typically developing (TD) controls. This study found that autistic individuals had increased grey matter volume in bilateral temporal and right lateral prefrontal cortices (Lukito et al., 2020). These structures are involved in social processes, including language processing and social interaction, which are impaired in ASD.

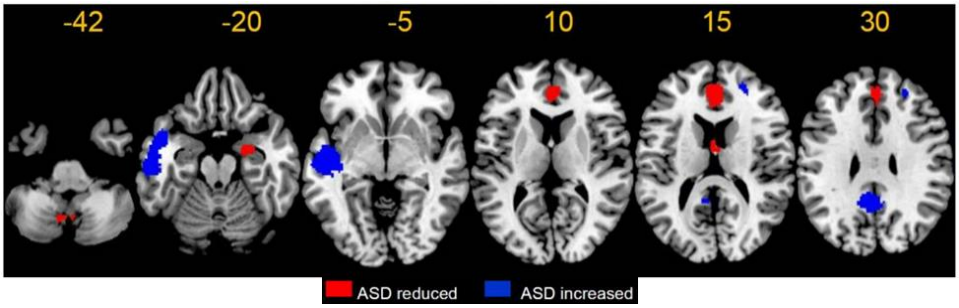


Figure 9. Structural comparison of ASD vs. typically developing brain.
Reproduced from Lutiko et al., 2020.

Functional MRI (fMRI) findings during cognitive control showed under activation in medial frontal regions, which are involved in sensory, emotional, and social processing, and overactivation in precuneus and bilateral ventrolateral regions (Lukito et al., 2020).

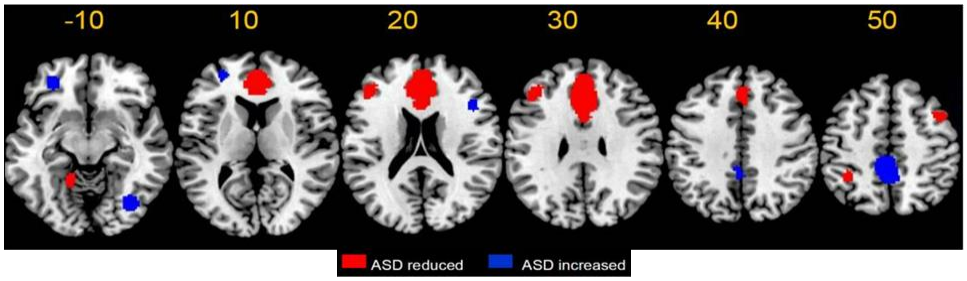


Figure 10. fMRI comparison of ASD vs. typically developing brains during cognitive control.
Reproduced from Lukito et al., 2020.

Findings during motor response inhibition showed ASD under activation in posterior regions and overactivation in left dorsal and ventral frontal regions (Lukito et al., 2020). ASD overactivation in the posterior cingulate cortex/precuneus indicates dysregulation of the default mode network (DMN), which is a prominent neurobiological feature of ASD (Padmanabhan et al., 2017). The DMN is significantly engaged during social cognitive tasks, including self-referential and autobiographical processing (Padmanabhan et al., 2017).

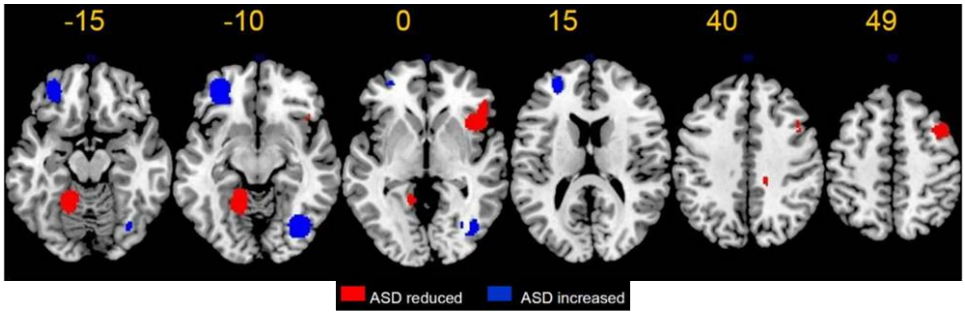


Figure 11. fMRI comparison of ASD vs. typically developing brains during motor response inhibition.
Reproduced from Lukito et al., 2020.

It is important to mention that structural and functional changes revealed by imaging in ASD are revealed across cohorts and populations, and not always, at the level of the individual.

ASD is not diagnosed by such MRI signals. Nevertheless, these studies garner insight into the alterations that are common across larger numbers of individuals as compared to matched neurotypical controls. Nevertheless, advances in neuroimaging, neuroscience, single cell approaches, and cellular/molecular biology have revealed phenomena that are likely important in neurodevelopmental disorder brain pathobiology and by extension, its etiologies.

In 2014, a study conducted by Stoner et al. used RNA in situ hybridization [with a panel of layer- and cell-type specific molecular markers] to phenotype cortical microstructure of children between the ages of 2 and 15 diagnosed with ASD and unaffected children. In situ hybridization is a technique to visualize expressed genes (mRNA transcripts) where they are expressed within this tissue itself. Ten of 11 children with ASD demonstrated focal "patches" of abnormal laminar cytoarchitecture and cortical disorganization of neurons (Stoner et al., 2014). These patches ranged from 5 to 7 mm in length and were adjacent to regions of cortex that appeared to be very similar to that of non-ASD children. Patches were present in the dorsolateral prefrontal cortexes (in 10 of 11 case samples) and posterior superior temporal cortex (in 2 of 2 case samples), but no patches were observed in the occipital cortex. The affected regions mediate functions that are disrupted in autism (i.e., social, emotional, language and communication functions). There was significant heterogeneity between case samples with respect to abnormal layers and cell types. Aberrant labeling was observed across consecutive sections, primarily characterized by decreased expression of specific cellular markers, which are typically present in fully differentiated cortical neurons, within localized regions. The most prominent indications of irregularity were found in cortical layers 4 and 5, but no layer was uniformly spared. Three-dimensional reconstruction of layer-specific markers was used to confirm patch size and distinct spatial arrangement.

The above finding of patchy disrupted cellular organization in ASD joins a larger literature in neurodevelopmental disorders showing mosaic developmental perturbations that support external stressors as contributors at key neurogenic timepoints of cortical development. This is not isolated to ASD, mosaicism is known in ADHD as well (Poliquin et al., 2021).

2. Altered Neurotransmission in ASD

Neurotransmitters are key chemical messengers in the brain. They are molecules that send signals between neurons, or neurons and muscles. Because of their importance in sending messages across different types of cells, neurotransmitters and neuropeptides are integral to brain development. They also impact neuronal cell migration, differentiation, synaptogenesis, apoptosis, and synaptic pruning (Marotta et al., 2020). Neurotransmitters also contribute to behavior, memory, and the regulation of motor activity (Marotta et al., 2020). Neurotransmitters are generally classified as small amine molecules, amino acids, or neuropeptides. Neuropeptides can have other functions outside of neural transmission and have regulatory functions including acting as signals and modulators outside of synapses.

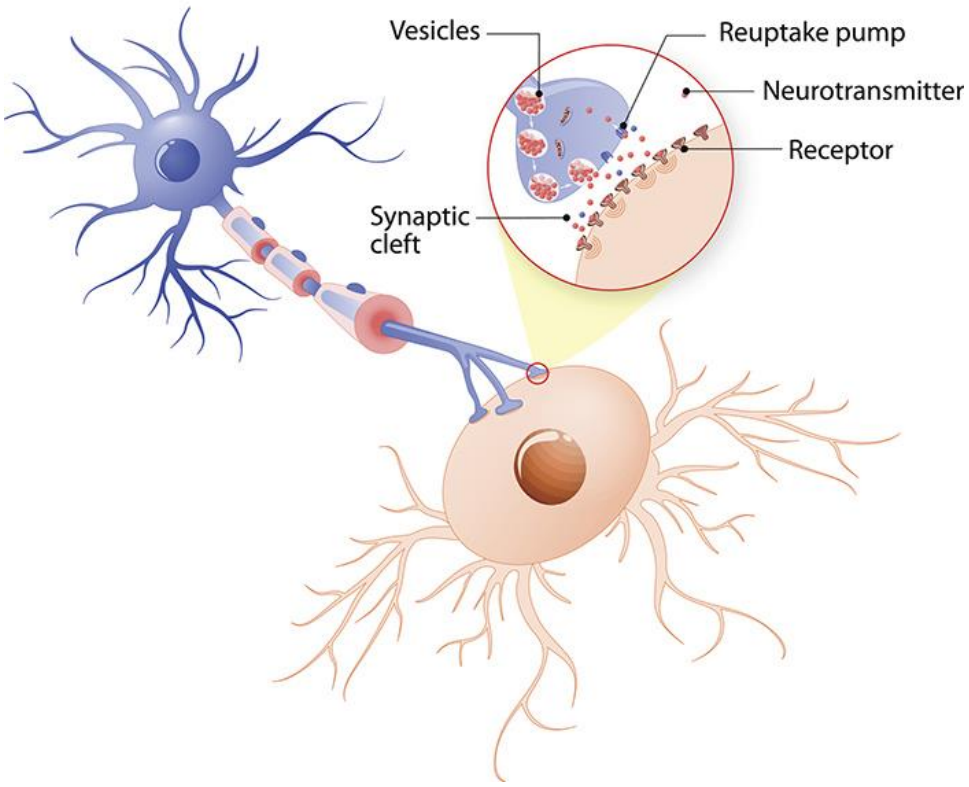


Figure 12. Neurotransmitters in action between two neurons.
Reproduced from iStockphoto.

While there are other known neurotransmitters and over 100 neuropeptides, the neurotransmitters and neuropeptides which contribute to the pathogenesis of ASD are:

Gamma aminobutyric acid (GABA) and glutamate. GABA (gamma-aminobutyric acid) and glutamate are the major inhibitory and excitatory neurotransmitters, respectively, in the central nervous system. They have complementary roles regulating neuronal activity and maintaining a balance between inhibition and excitation. GABA plays a crucial role in the development of the brain by regulating neuronal migration, promoting synapse formation, and influencing the establishment of neuronal circuits, thereby contributing to the refinement of neural connectivity and the proper functioning of the developing brain (Marotta et al., 2020). Children with ASD have altered levels of plasma GABA and glutamate, and adults with ASD exhibit decreased levels of glutamate in the striatum (Horder et al., 2018; Marotta et al., 2020). Additionally, magnetic resonance spectroscopy studies have revealed age-dependent reductions in GABA in various brain regions such as the motor, visual, auditory, and somatosensory areas, as well as the left hemisphere's perisylvian region, which can contribute to abnormal information processing (Marotta et al., 2020; Puts et al., 2017; Rojas et al., 2014).

Dopamine. Dopamine is a critical neurotransmitter that can modulate a wide range of functions within the brain, including movement, motivation, reward, mood, attention, and learning. Several studies have demonstrated linked dopaminergic dysfunction in specific brain structures to ASD (Dichter et al., 2012; Ernst et al., 1997; Scott-Van Zeeland et al., 2010). Specifically, the social deficits exhibited by persons diagnosed with ASD are linked to changes in the

mesocorticolimbic dopaminergic signaling pathway, which is involved in reward and motivation-related behavior (Marotta et al., 2020; Paval, 2017; Paval & Miclutia, 2021). Studies on animal models of ASD indicate that the dysfunction of the nigrostriatal dopaminergic pathway leads to stereotyped behaviors and may impact social behavior, as well (Lewis et al., 2006, Marotta et al., 2020; Paval 2017; Paval & Miclutia 2021). Genetic studies of ASD have found several gene polymorphisms related to dopaminergic pathways, including dopamine receptors DR3 and DR4 and dopamine transporter (DAT) (Marotta et al., 2020).

Serotonin. Serotonin is another critical neurotransmitter involved in numerous brain functions, including memory, learning and the regulation of mood and sleep. The serotonergic system and its effects are revisited later in this report, but several neurodevelopmental processes are impacted by serotonin, including cell division, cortical proliferation, migration, differentiation, cortical plasticity and synaptogenesis (Marotta et al., 2020, Celada et al., 2013). Typically developing children experience elevated levels of serotonin synthesis between two and five years of age, which declines at puberty; however, children diagnosed with ASD experience significantly lower levels of serotonin at two to five years, which increases with age, and do not experience decreased serotonin synthesis over time (Marotta et al., 2020).

Acetylcholine. Acetylcholine is another important neurotransmitter present in both the central and peripheral nervous system (PNS). Acetylcholine, the first neurotransmitter to be discovered, is involved in several physiological processes, including cognition, memory, and regulation of the parasympathetic nervous system (Marotta et al., 2020). Post-mortem studies have shown autistic individuals have significantly reduced nicotinic α4β2 acetylcholine receptors in the frontal and parietal cortex (Perry et al., 2001) and reduced α4 acetylcholine receptors in the cerebellum, which may be related to Purkinje cell loss and a compensatory increase in α7 acetylcholine receptors (Lee et al., 2002).

Other neurochemical systems have varying levels of evidence for involvement in ASD including N-acetyl aspartate, oxytocin and arginine-vasopressin, melatonin, vitamin D, orexin, and endogenous opioids (Marotta et al., 2020.)

B. ATTENTION-DEFICIT/HYPERACTIVITY DISORDER

The DSM-5 defines Attention-Deficit/Hyperactivity Disorder (ADHD) as a neurodevelopmental disorder characterized by a persistent pattern of inattention and/or hyperactivity-impulsivity that interferes with functioning or development, as manifested by both of the following:

1. Inattention: Six or more of the following symptoms of inattention have been present for at least six months to a degree that is inconsistent with developmental level and that negatively impacts directly on social and academic/occupational activities:

    a. Fails to give close attention to details or makes careless mistakes in schoolwork, work, or other activities
    b. Has difficulty sustaining attention in tasks or play activities
    c. Does not appear to listen when spoken to directly

d. Does not follow through on instructions and fails to finish schoolwork, chores, or duties in the workplace
e. Has difficulty organizing tasks and activities
f. Avoids, dislikes, or is reluctant to engage in tasks that require sustained mental effort
g. Loses things necessary for tasks or activities
h. Is easily distracted by extraneous stimuli
i. Is forgetful in daily activities

2. Hyperactivity and Impulsivity: Six or more of the following symptoms of hyperactivity-impulsivity have been present for at least six months to a degree that is inconsistent with developmental level and that negatively impacts directly on social and academic/occupational activities:

a. Fidgets with or taps hands or feet or squirms in seat
b. Leaves seat in situations when remaining seated is expected
c. Runs about or climbs in situations where it is inappropriate
d. Unable to play or engage in leisure activities quietly
e. Is "on the go" or often acts as if "driven by a motor"
f. Talks excessively
g. Blurts out an answer before a question has been completed
h. Has difficulty waiting for their turn
i. Interrupts or intrudes on others (e.g., butts into conversations or games)

The symptoms must have been present prior to age 12 and must be present in two or more settings (e.g., at home, school, or work). Additionally, several other criteria must be met for a diagnosis of ADHD, including evidence that the symptoms interfere with social, academic, or occupational functioning and the symptoms are not better explained by another mental disorder. The DSM-5 recognizes three presentations of ADHD: predominantly inattentive, predominantly hyperactive-impulsive, and combined presentation.

1. Structural and Functional Brain Abnormalities in ADHD

Consistent with the heterogenous clinical presentation of ADHD, imaging studies demonstrate variability in the changes to neurological structures and functions linked with ADHD. Voxel-based morphometry meta-analyses have shown reduced grey matter volume in basal ganglia and ventromedial orbitofrontal cortex in ADHD (Nakao et al., 2011; Frodl et al., 2012; Norman et al., 2016). A mega-analysis performed by the ENIGMA consortium found individuals with ADHD have reduced grey matter volume in the basal ganglia, amygdala and hippocampus and reduced cortical surface area and thickness in frontal, temporal, and parietal regions (Hoogman et al., 2017; Hoogman et al., 2019). The meta-analysis performed by Lukito et al. demonstrated reduced grey matter volume in the ventromedial orbitofrontal cortex in ADHD, which may be related to the dysfunction of reward-based decision-making neural networks (Lukito et al., 2020).

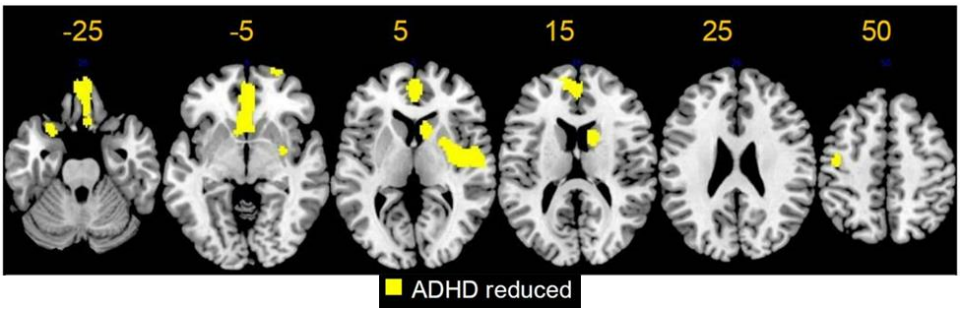


Figure 13. Structural comparison of ADHD vs. typically developing brain. Reproduced from Lutiko et al., 2020.

Some volumetric changes observed in childhood normalize later in life, but the changes in many measures remain fixed. For example, a longitudinal study of ADHD patients showed that reduced dorsal surface area and lower basal ganglion volumes shown in adolescence did not normalize with aging (Faraone et al., 2015). Additionally, ADHD subjects experienced a progressive contraction of ventral striatal surface area with aging, whereas control subjects experienced increased surface area with age (Faraone et al., 2015).

"Remission of ADHD has been associated with normalization of abnormalities as measured by activation during functioning imaging tasks, cortical thinning, and structural and functional brain connectivity. Although these data could be taken to suggest that the age-dependent decline in the prevalence of ADHD might be due to the late development of ADHD-associated brain structures and functions, most patients with ADHD do not show complete developmental 'catch up'. Indeed, widespread deviations in cortical thickness persist in many adults with ADHD" (Faraone et al., 2015).

With respect to functional differences in ADHD compared to typically developing controls, meta-analyses demonstrate underactivation in lateral and frontostriatal networks (i.e., right anterior cingulate cortex/anterior insula/inferior frontal gyrus/supplementary motor area, and striatum) (Hart et al., 2013; Norman et al., 2016; Lukito et al., 2020).

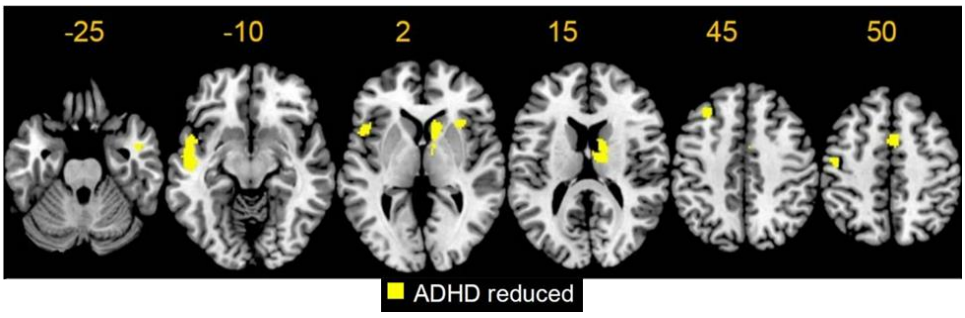


Figure 14.fMRI comparison of ADHD vs. typically developing brains during cognitive control. Reproduced from Lutiko et al., 2020.

The meta-analysis by Lukito revealed ADHD underactivation in the right inferior frontal and striatal regions during motor response inhibition. This finding was consistent with previous findings of underactivation in the right inferior frontal gyrus and caudate, which are key to

cognitive control processes including attention, decision-making, and inhibitory control (Hart et al., 2013; Rae et al., 2014; Norman et al., 2016; Zhang et al., 2017; Lukito et al., 2020).

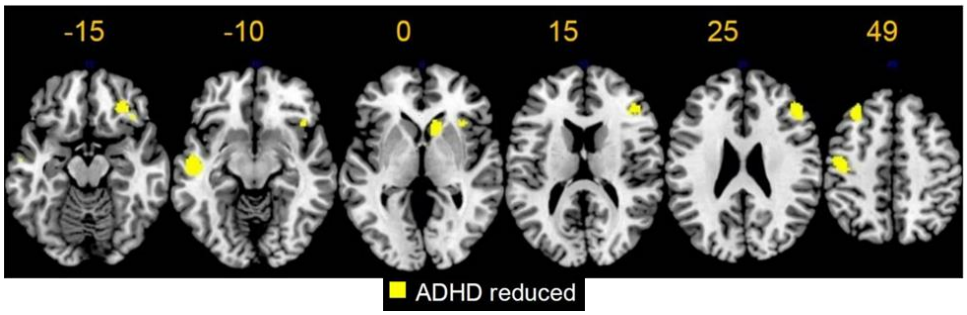


Figure 15. fMRI comparison of ADHD vs. typically developing brains during motor response inhibition. Reproduced from Lutiko et al., 2020.

Baker et al., 2020 studied the association between prenatal APAP exposure, determined by presence and level of APAP in meconium, and ADHD in children. The potential for mediation of the association between prenatal APAP exposure and hyperactivity was tested by reference to resting-state brain activity from fMRI. Compared to children with no APAP detected in meconium (n = 23), children who had detectible levels of meconium APAP (n = 25) showed increased negative connectivity between the medial prefrontal cortex gyrus and regions of bilateral precentral and postcentral gyri, superior parietal lobules, and supramarginal gyri, as well as increased negative connectivity between the left lateral prefrontal cortex and portions of the right precentral and frontal gyrus (Baker et al., 2020). These findings involve many of same regions identified in meta-analyses of structural and functional MRI of ADHD.

2. Altered Neurotransmission in ADHD

Genome-wide association studies have found several genetic variants involving neurotransmission and neurodevelopment are implicated in ADHD. This data, which provides important information about ADHD etiology and pathophysiology, identifies six primary neurotransmitters/neuroreceptors that play an important role in the pathophysiology and pathogenesis of ADHD: dopamine, serotonin, norepinephrine, acetylcholine, and glutamate/GABA.

Dopamine. The dopaminergic pathway plays a vital role in the development and manifestation of ADHD. Dopamine signals regulate emotion, reward, locomotion, complex behavior, and cognition, and dysfunctional dopamine signaling in the forebrain leads to inappropriate or deficient attention (Kessi et al., 2022). Numerous studies have shown that persons with ADHD have decreased dopamine receptor density in several brain regions, and studies of children with ADHD have identified the frontal cortical regions, which are rich in dopamine, as sites related to ADHD (Kessi et al., 2022).

Serotonin. Patients with ADHD have shown relatively low platelet serotonin levels. Serotonin signaling regulates cognition, behavior, and immunity, and disrupted serotonergic development can alter brain function resulting in anxiety, depression, impulsivity, irregular appetite, and violence (Corominas-Roso et al., 2013). Biochemical and pharmacological evidence

demonstrate that reduced serotonergic function is linked to impulsivity, which is a prominent feature in ADHD (Kessi et al., 2022).

Norepinephrine. Adults diagnosed with ADHD have shown reduced norepinephrine availability in brain regions relevant to attention, which is more pronounced in the right hemisphere (Ulke et al., 2019). Norepinephrine signaling regulates cognition, memory, and stress regulation.

GABA and Glutamate. As discussed above, Glutamate is involved in several neurological functions, including synaptic plasticity, regulating dopamine signaling, memory formation, learning, and brain development, (Kessi et al., 2022). Children with ADHD have shown higher levels of glutamate and lower levels of GABA compared to controls, and this imbalance leads to inattention, hyperactivity, and impulsivity (Courvoisie et al., 2004).

Acetylcholine. Cholinergic dysfunction is associated with many neurological conditions, including ADHD (English et al., 2009). Acetylcholine is critical to the regulation of several CNS functions, including attention, learning, memory and motivation – all of which are dysregulated in persons with ADHD (Johansson et al., 2013).

C. ASD AND ADHD SHARED CHARACTERISTICS

ASD and ADHD share some symptomology and are often diagnosed together. A significant proportion of children with ASD also meet the criteria for ADHD. Both ADHD and ASD are comprised of symptoms characterized by difficulties with attention and social interaction. Children diagnosed with ADHD may have difficulty focusing on tasks, may be easily distracted, and may struggle with impulse control. Similarly, children with ASD may have difficulty with attention and may exhibit repetitive behaviors or restricted interests. Additionally, both disorders can lead to problems with social interaction and communication. Children with ADHD may struggle to notice social cues or may interrupt others during conversations. Children with ASD may have difficulty understanding social norms or may struggle to make eye contact during conversations.

One hallmark of both ASD and ADHD is sex-specific differences in the way each disorder typically manifests. These sex-specific features are present in both humans and their corresponding animal models (Berger & Sagvolden, 1998; Jeon et al., 2018; Mahendiran et al., 2019). The complete mechanisms of such sex differences are not fully characterized but can reflect hormonal, genetic, societal, clinical, and even immunological differences.

D. SHARED ETIOLOGY OF NEURODEVELOPMENTAL DISORDERS

As discussed above, ASD and ADHD are often co-diagnosed, and research has identified shared genetic and environmental factors that contribute to the development of both ASD and ADHD. ASD and ADHD are both individual phenotypes that may have a wide range of overlapping etiologies, including genetic and environmental factors.

Several genes have been identified that are associated with an increased risk for both ADHD and ASD, including genes involved in neurotransmitter signaling and neuronal development (Satterstrom et al., 2019; Mattheisen et al., 2022). However, as discussed in more

detail later in this report, the presence of a gene, or group of genes, which create a propensity for ASD and/or ADHD is not the sole determiner of the ultimate phenotype, meaning that many people carry variants in these genes and are ultimately not diagnosed with either disorder.

Environmental factors, such as prenatal exposure to toxins and toxicants, have also been linked to an increased risk for both disorders. Brain imaging studies and neurochemical analysis have also identified significant shared abnormalities in individuals with ADHD and ASD, particularly in regions of the brain involved in social cognition, attention, and executive functioning (see, e.g., Rommelse et al., 2010). Analyses of the effect of APAP on neurodevelopment have also specifically pointed out that shared environmental risk factors for ADHD and ASD may have a transdiagnostic feature (Kim et al., 2020). These findings suggest that there may be common underlying neural mechanisms that contribute to the development of both disorders.

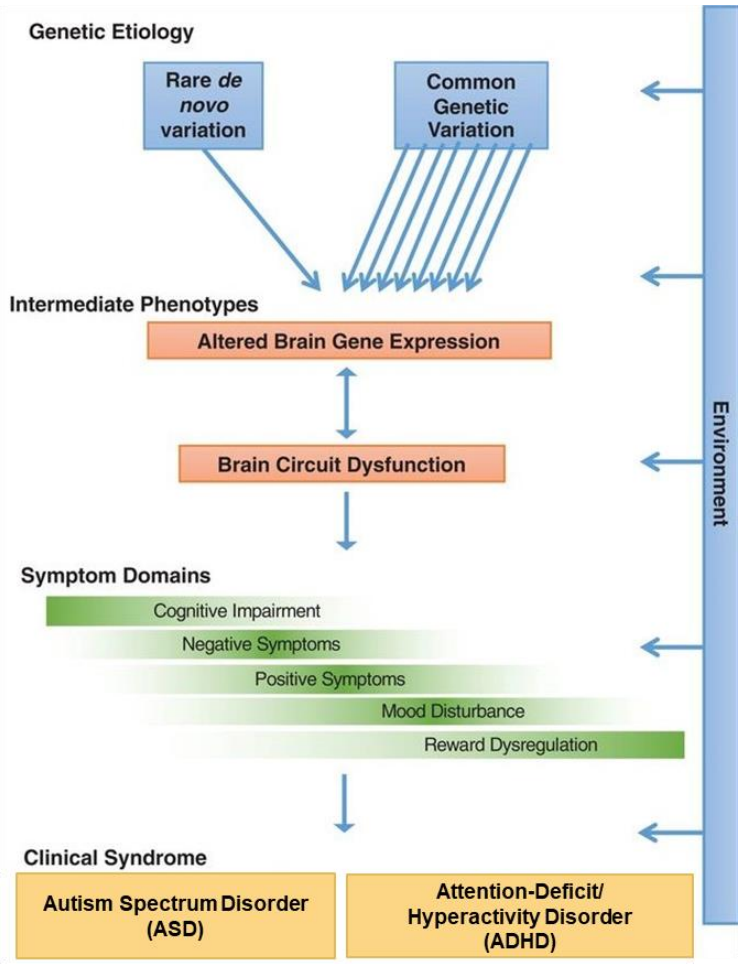


Figure 16. Depiction of how genetic and environmental etiological factors (blue boxes) converge on molecular, cellular and synaptic "intermediate phenotypes" that underlie symptom domains and clinical phenotypes that overlap, cellular and synaptic "intermediate phenotypes" that underlie symptom domains and clinical phenotypes that overlap and share upstream contributors.
Modified from Gandal et al., 2018.

E. GENETIC SUSCEPTIBILITY TO NEURODEVELOPMENTAL DISORDERS

1. Genes and Gene Expression

As discussed above, genes play an important role in determining whether an individual person may develop a neurodevelopmental disorder. However, gene expression, a delicate and easily disrupted process, is perhaps more central. Genes and gene expression are two distinct but closely related concepts.

Genes themselves are the segments of DNA that carry hereditary information for protein sequences. Variation in gene sequence affects protein sequences capable of contributing to pathological disease states, or vulnerabilities to disease states. Gene variants can be inherited or they can emerge in parental germ cells (de novo mutations) or even arise post conception in what are termed somatic mosaic mutations.

Gene expression encompasses the complex processes by which the information within a gene is used to generate functional products, often a protein or RNA molecule. While an individual organism has many genes, the only observable traits or characteristics that are ultimately expressed are phenotypes.

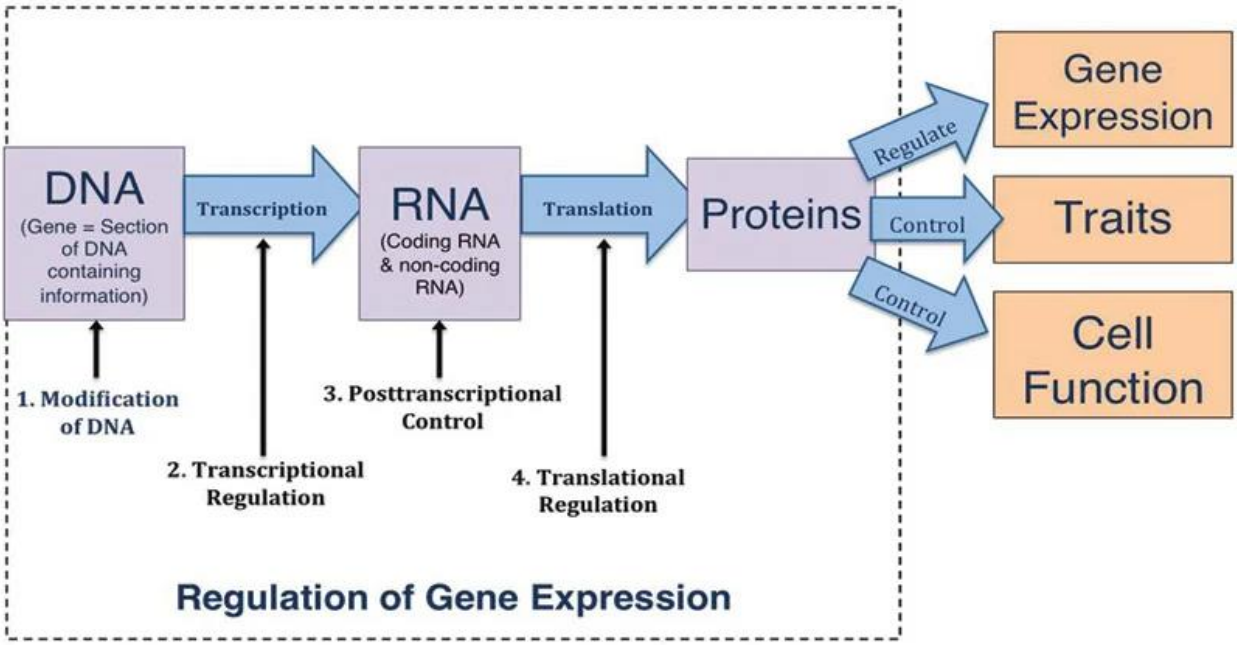


Figure 17. Graphical depiction of how the information molecules of a cell ensure that a cell does what it is supposed to do under a normal, basal state, but also can respond to stimuli as needed, in an adaptive manner. Reproduced from https://microbenotes.com/gene-expression/.

Phenotypes are the outward manifestations of the underlying genetic and environmental influences on an organism. While an individual organism may have any number of underlying genes, the ultimate outcome for that organism (the phenotype) is determined through a combination of gene

expression and other environmental factors. Gene expression is tightly regulated to ensure that specific genes are activated or repressed in a precise and coordinated manner. This regulation allows cells to respond to diverse signals and adapt to environmental changes. Gene expression control occurs at multiple levels, including transcriptional regulation through interactions between transcription factors and gene promoters or enhancers. types. The timing and way genes are expressed allow the diverse array of cell types and tissues to form in order generate an organism with its component parts. Flexibility in gene expression programs is necessary to respond to environmental and homeostatic changes, but disturbances to adaptative and normal developmental transcriptional programs can underlie pathologies independent of, or in ways that interact with gene sequence.

Gene expression involves sequential steps. Gene expression begins with transcription, wherein a complementary RNA molecule, known as messenger RNA (mRNA), is synthesized from a DNA template. Within our cells, mRNA's role is to carry genetic information from DNA to the ribosomes, where it serves as a template for protein synthesis. Once the mRNA molecules carry the genetic code from the nucleus to the cytoplasm, translation begins. During translation, ribosomes and transfer RNA (tRNA) molecules facilitate the synthesis of a protein by interpreting the mRNA template. Once the process of transcription is complete, regulation continues through mechanisms such as microRNAs, which modulate mRNA translation. Post-translational modifications, such as phosphorylation or acetylation of proteins, can also influence gene expression.

Gene expression refers to the process of converting genetic information into functional molecules. By contrast, genetic susceptibility refers to the increased likelihood of developing a trait or disease (i.e., a phenotype) based on specific genetic variations. Genetic susceptibility can be influenced by genetic variations, including mutations or polymorphisms in specific genes or regions of the genome. These variations can impact an individual's susceptibility to various disorders, including neurodevelopmental disorders, and individual toxic exposures.

As the diagram adapted from Gandal et. al. (Fig 16) illustrates, altered gene expression disrupts normal brain circuitry. That disruption may manifest in phenotypes or behaviors that fall into any number of categories in terms of a formal (human) diagnosis. As discussed above, although the diagnostic criteria in humans have a place, they are fundamentally a clinical construct with limited utility in terms of determining the etiology of a specific disorder, particularly for highly heterogeneous disorders like ASD and ADHD.

With regard to APAP and gene expression during neurodevelopment in particular, there is ample evidence that APAP disrupts the delicate process of gene expression in the developing brain (see, e.g., Baker et al., 2023).

2. Specific Syndromes

Neurodevelopmental syndromes are a distinct subset of neurodevelopmental disorders characterized by specific clinical features and associated genetic abnormalities. These syndromes, such as Down syndrome, Fragile X syndrome, Rett syndrome, and Angelman syndrome, among others, are characterized by well-defined, highly penetrant genetic etiologies and often exhibit unique clinical presentations. Unlike ASD or ADHD, neurodevelopmental syndromes typically

demonstrate less heterogeneity and are distinguished by their genetic causes and clinical profiles. Syndromes are generally distinct in that the etiology or cause of the disease or disorder is well defined.

3. Neurotoxins and Neurotoxicants

Neurotoxins and neurotoxicants are substances that can harm or impair the development and function of the nervous system. The terms neurotoxin and neurotoxicants are often used interchangeably but have slightly different meanings depending on the context Neurotoxicologists commonly differentiate between substances that have biological origins as neurotoxins (such as BOTOX or Botulinum toxin which is a chemical derived from bacteria) versus toxicants or neurotoxicants, used more broadly (and which will be used hereafter) to also include those that are synthetic or result from anthropogenic activities, such as pesticides, industrial chemicals, or drugs. Neurotoxicants can damage the nervous system at any age, but exposure during critical periods of neurodevelopment can disrupt normal brain development, leading to an increased risk of neurodevelopmental disorders. Substances that are particularly harmful during periods of neurodevelopment are sometimes referred to as developmental neurotoxins or developmental neurotoxicants.

The neurodevelopmental disorders caused by toxic substances encompass a wide range of conditions characterized by atypical brain development, altered neuronal connectivity, and subsequent cognitive, behavioral, and functional impairments.

Various environmental factors contribute to neurotoxicant exposure, including industrial pollutants, heavy metals (e.g., lead, mercury), pesticides, certain medications, and maternal substance abuse. The developing brain is particularly vulnerable to the adverse effects of neurotoxicants as it undergoes intricate processes of neuronal proliferation, migration, cell fate determination, synaptogenesis, and myelination.

Neurotoxicants can exert harmful effects through multiple mechanisms. They may interfere with neurotransmitter systems, disrupt synaptic connections, alter cellular signaling pathways, induce oxidative stress and inflammation, and interfere with the balance of essential neurodevelopmental processes. These disruptions can result in long-lasting or permanent changes in brain structure and function, contributing to the development of neurodevelopmental disorders.

VII. PRECLINICAL RESEARCH

Preclinical studies are laboratory studies that include animal models (in vivo), cell culture (in vitro), computational (in silico), and ex vivo (organs or tissues studies outside of the body). In general, preclinical studies are performed prior to human clinical trials to understand the efficacy and/or toxicity of a device or compound. However, preclinical research is also performed when the safety of an approved or existing compound is unclear or called into question. Preclinical research is essential to prevent waste of financial and clinical trial resources and is performed in lieu of invasive or unethical human experimental approaches to avoid side effects or other serious adverse effects of test agents, drugs, or devices and for approved substances whether they are agrochemicals, industrial agents, approved therapeutics, or legacy drugs or chemicals. Subtypes of Preclinical Research include:

In Vivo. In vivo means experiments done in or using the whole body of a whole organism. A drug administered "in vivo" means it is given systemically to the intact animal. A main benefit of in vivo preclinical research is that it models the human condition most directly and can evaluate the systemic effects across organ systems as well as health and behavioral influences. In biomedical pursuits, we are attempting to understand how chemicals make us sick or healthy. An intact organism with its varied and interconnected systems (circulatory, endocrine, nervous, gastrointestinal, etc.) will always be the most ideal way of understanding the full impacts of a drug. However, ethics considerations are such that we always want to reduce, refine, and replace in vivo animal model approaches where we can. Animal models are discussed in more detail later in this report.

In Vitro. In vitro means in culture, or cultured cells or tissue that are grown in plates or dishes in an incubator, outside of the body. Often immortalized cell lines (i.e., cells that have been manipulated to proliferate indefinitely) are used as they are robust and well characterized. Primary cells are cells that are harvested directly from tissues and grown. The benefits of in vitro experiments are that researchers can use relevant cell types but avoid issues surrounding drug delivery. They also reduce the numbers of animals used in preclinical research and create opportunities for higher dimensionality for doses, duration, chemicals, and readouts. There are instances where in vitro approaches will not be sufficient.

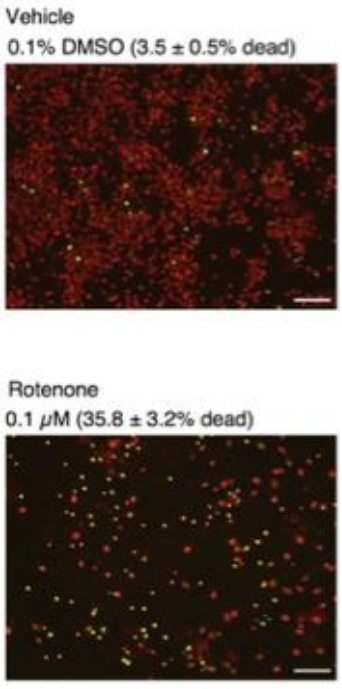


Figure 18.

Cell viability or the inverse, cell death (as in the Figure on the left which shows primary cortical neurons grown from embryonic mouse brains [data from Pearson et al., 2016 *Nature Communications]*) can be evaluated in vitro. The growth of the cells and function of the cells can be measured. Other metrics such as enzyme activities, or in the case of nervous system cells, electrochemical communication or synapse formation are readily evaluated in cultured cells. The advent of human induced pluripotent stem cell (iPSC) technologies has created the opportunity to grow human induced stem cell-like cells from human participants which can be tested for toxicological effects as stem cells or in terminally differentiated cell types such as neurons. In vitro technologies are rapidly advancing, but they do not replicate the entirety of an intact organism with other organ systems (circulatory system, biliary system, renal system, immune system, etc). Toxicants and drugs do not act in a vacuum of one cell type or one system so in vitro testing must consider its inherent limitations.

In Silico. In silico means computational. These are analyses using a computer that are used to predict properties of chemicals where data from physical experiments is lacking or non-existent. Read across tools can be used to fill data gaps about chemical toxicity for assay outcomes where they have not actually been tested. Using quantitative structure activity relationships (QSAR), physical-chemical properties, and other variables can be used to in regressions and machine learning algorithms to classify and predict toxicological endpoints for thousands of environmental chemicals and drugs. Bioinformatics tools, data visualizations, data reduction techniques, data integration and other

statistical methods are also computational or in silico approaches that can aid in determining the toxicity of a chemical.

VIII. BIOLOGICAL PLAUSIBILITY

The preclinical studies reviewed in this report relate to the biological plausibility that APAP causes neurodevelopmental disorders. Biological plausibility concerns whether, based on existing knowledge about human biology and disease pathology, an exposure could plausibly cause a disease. Biological plausibility is one of the factors enumerated by Sir Austin Bradford Hill to aid epidemiologists, who study the incidence of disease in human populations, in determining causation.[5] In this case, numerous observational human studies (i.e., epidemiological studies) have found a consistent association between APAP exposure in utero and neurodevelopmental disorders, most frequently ASD and ADHD (See expert report of Andrea Baccarelli, MD, PhD, MPH). Those studies include multiple careful study design features designed by researchers to make the results as clinically relevant as possible and to reduce confounding, but they do contain limitations as described by their authors (and as typically required by the peer review process) (See expert report of Andrea Baccarelli, MD, PhD, MPH).

Though the most conclusive answer to the causation inquiry would be obtained through a randomized controlled trial (RCT), a RCT would not be feasible in this situation for several reasons. Most importantly, it would be unethical to subject pregnant women to treatment with a drug suspected to cause neurodevelopmental disorders in offspring. Causation can be established in the absence of randomized control trials (see, e.g., the causal link between smoking and lung cancer and newer causal epidemiological methods [e.g., Mendelian randomization, reviewed in Sanderson et al., 2022]). Biologic plausibility based on preclinical studies plays an especially

---

The Bradford-Hill considerations provide a framework within epidemiology to assess causation and include:

1. **Strength** (effect size): A small association does not mean that there is not a causal effect, though the larger the association, the more likely that it is causal.
2. **Consistency** (reproducibility): Consistent findings observed by different persons in different places with different samples strengthens the likelihood of an effect.
3. **Specificity**: Causation is likely if there is a very specific population at a specific site and disease with no other likely explanation. The more specific an association between a factor and an effect is, the bigger the probability of a causal relationship.
4. **Temporality**: The effect has to occur after the cause (and if there is an expected delay between the cause and expected effect, then the effect must occur after that delay).
5. **Biological gradient**: Greater exposure should generally lead to greater incidence of the effect. However, in some cases, the mere presence of the factor can trigger the effect. In other cases, an inverse proportion is observed: greater exposure leads to lower incidence.
6. **Plausibility**: A plausible mechanism between cause and effect is helpful (but Hill noted that knowledge of the mechanism is limited by current knowledge).
7. **Coherence**: Coherence between epidemiological and laboratory findings increases the likelihood of an effect. However, Hill noted that "... lack of such [laboratory] evidence cannot nullify the epidemiological effect on associations".
8. **Experiment**: "Occasionally it is possible to appeal to experimental evidence".
9. **Analogy**: The effect of similar factors may be considered.

critical role in situations where such testing is impossible or unethical, and an essential role even when RCT is possible.

Because observational human studies do have limitations, animal models and other preclinical studies are critical and commonly used to determine causation. While epidemiological studies may have hidden confounders, researchers performing preclinical tests in animals exert substantial control over all variables and thereby mitigate confounding and bias concerns. They can also help show the plausible mechanisms of action for a particular injury.

As described below, the preclinical research relevant here demonstrates the biological plausibility that prenatal APAP exposure causes neurodevelopmental disorders, including ASD and ADHD, in offspring.

IX. ANIMAL MODELS IN PHARMACEUTICAL AND NEURODEVELOPMENTAL RESEARCH

In vivo studies—in other words, animal studies—are a primary source for preclinical data of biological possibility, including for studying ASD and ADHD. Although these are human disorders, other animals, such as rats and mice, express analogous symptoms as humans with those disorders. Thus, the disorders can be "modeled" in these animals, enabling researchers to study potential causes of these symptoms.

The phrase "animal model" refers to the use of non-human animals to replicate key features of human diseases, conditions, or related physiological processes. These models are created by genetic manipulation, selective breeding for naturally occurring characteristics that resemble human disease, or exposure to environmental factors or inducing lesions that mimic human disease (Wickens et al., 2011). The validity of a proposed animal model is evaluated primarily by reference to three criteria: (1) face validity (i.e., the degree to which the model mimics the fundamental behavioral characteristics of the human disorder); (2) construct validity (i.e., the degree to which the animal expresses the known etiology and pathophysiology of the human disorder); and (3) predictive validity (i.e., the degree to which the animal's response to treatment corresponds to human treatment response) (Belzung et al., 2011). Rodent models of ASD, for instance, can demonstrate good face validity, moderate to good construct validity, but cannot demonstrate good predictive validity because there are no curative treatments that resolve the symptoms of ASD that can be taken to the animal model to validate it. This does not invalidate their use, but it does provide an example of how substantial expertise is required in designing and using rodent models of neurodevelopmental disorders and in their interpretation.

Animal models offer several advantages in the study of human diseases, allowing researchers to efficiently test hypotheses, control genetic and environmental influences, and perform invasive studies in a manner that is not feasible or ethical in humans. Accordingly, animal models are frequently used in scientific research, including toxicological research, to investigate the causes, mechanisms, progression, and potential treatments of disease. Animal studies have played a key role in identifying the harmful neurodevelopmental effects of drugs already on the market, such as Valproic Acid. Indeed, the FDA specifically looks to animal data to demonstrate the effectiveness of a drug when adequate and well-controlled efficacy studies in humans cannot be ethically conducted (FDA CDER, 2015 "Animal Rule").

Many species can be used to model human disease, and the selection of test species generally depends on several factors (e.g., specific research question, neurobiological system of interest, desired endpoint). Humans and other primates share the vast majority of their genetic information, but primate models are relatively uncommon because they are costly, not widely available, and ethically dubious. Rodents and other small mammals are less resource-intensive and more susceptible to genetic modifications commonly used to model human disease. Rats and mice are frequently used in animal experimentation because they are easy to maintain in the laboratory and can be bred rapidly and at relatively low cost. From a practical standpoint, mice are less expensive and take up less space than larger mammals, allowing researchers to design studies with larger samples. Mice are also easier to genetically manipulate, allowing researchers to perform studies on mice with or without specific genetic characteristics. Rats, on the other hand, typically display more complex social behaviors and therefore can help researchers understand more complex effects of specific compounds. Both rats and mice share a high degree of genetic and physiological concordance with humans. In fact, humans and laboratory rodents are genetically very similar: the genome of humans and mice are approximately 85% identical (Why Mouse Matters, National Human Genome Research Institute, available at https://www.genome.gov/10001345/importance-of-mouse-genome).

Animal models play a crucial role in advancing our understanding of neurodevelopmental disorders, including ASD and ADHD, which are caused by the complex interaction of genetic, epigenetic, and environmental factors. By inducing genetic mutations or exposing animals to toxicants believed to impact neurodevelopment, scientists can observe changes to a myriad of components of the central nervous system, including its neuroanatomical structures, synaptic connectivity, and neurochemical signaling. Animal models can also provide critical information about potential therapeutic interventions and help researchers identify targets for pharmacological interventions. While animal models do not perfectly replicate the complexity of human conditions, they offer a valuable experimental platform for studying neurodevelopmental disorders and aid in the development of new treatments and interventions.

A. ANIMAL MODELS OF ASD

Animal models have been widely used to study ASD. Animal research related to ASD has included research into both the etiology of autism phenotypes (i.e., cause of ASD symptoms) as well as potential targets for pharmacological treatments. Researchers use a variety of animal models, including flies, fish, mice, rats, voles, and non-human primates to mimic the genetic, environmental, and behavioral aspects of ASD in humans. These models allow researchers to ethically study the underlying genetic factors, molecular pathways, and environmental impacts that contribute to ASD. Despite the diversity of species used, mouse models predominate.

The core symptoms of ASD modeled in animals are in social and self-directed behavioral domains. Social behaviors are generally evaluated in social motivation and social communication paradigms; self-directed behaviors are generally evaluated by evaluating repetitive or restricted motor behaviors. Individuals diagnosed with ASD often engage in repetitive stereotyped behaviors. Many mouse models of ASD also engage in repetitive stereotyped behaviors. This can be evaluated by evaluating motor movements such as circling behaviors, the patterns and durations of body grooming behaviors, and the manners in which mice interact with the cages or with novel

objects (Pearson et al., 2010). Several of the most common tests, which are used in several of the studies discussed in this report, are described below.

1. Animal Behavioral Testing and ASD

a. Social Behavior

"Mice have a natural tendency to approach and investigate unfamiliar conspecifics, much like the way a person would greet a stranger. A person with autism often avoids interacting with a stranger, either withdrawing when approached or remaining aloof. Therefore, low social approach in mice represents an endophenotype with a reasonable analogy to the types of social deficits that characterize autism" (Yang et al., 2011).

Three-Chamber Social Test. The three-chamber social interaction test, developed by Dr. Jackie Crawley, a pioneer in behavioral neuroscience, is commonly used to measure social interest and social novelty seeking behavior (Yang et al., 2011). In this test, the subject rodent explores a partitioned arena with a neutral central chamber and two side chambers containing stimuli, as depicted in Figure 19. The sociability phase measures the time spent in the chamber containing a caged rodent versus the chamber containing an empty cage. The social novelty phase measures the time spent in the chamber containing a novel rodent versus the chamber containing the familiar rodent (Bey & Jiang, 2014). Failing to spend more time in the chamber with the mouse vs the empty cage side indicates low interest in peers (associated with ASD's DSM-IV factor I). Likewise, a failure to spend more time in the chamber containing the unfamiliar (novel) rodent compared to the familiar rodent during the social novelty phase indicates a lack of social recognition or social memory (also linked to ASD factor I).

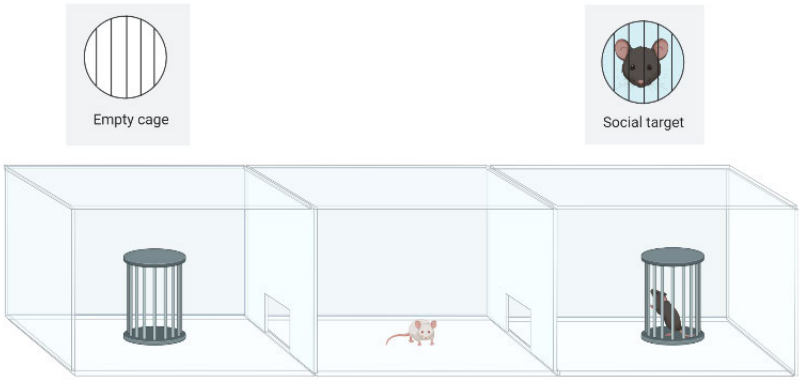


Figure 19. Three-chamber sociability arena.
Created with Biorender.

Direct Interaction Tests. In this test the subject rodent freely interacts with a stimulus rodent and the number, duration, and types of social interactions are measured. Variations of this test include neutral pairing, i.e., the stimulus rodent is an ovariectomized female or juvenile male; or agonistic pairing, i.e., the stimulus rodent is an adult male in the novel arena social (social dyadic) or test rodent's home cage (resident intruder). (Bey & Jiang, 2014). A low number of

interactions and/or little time spent interacting indicate low interest in peers and/or difficulty in maintaining interaction (ASD factor I).

Habituation-Dishabituation Tests. The subject rodent is repeatedly introduced to the stimulus rodent. Decreasing time spent in social interaction represents social recognition of the stimulus rodent. Then, a novel rodent can be introduced as a stimulus rodent, and increased time spent in social interaction represents social recognition. (Bey & Jiang, 2014). Impaired social memory and recognition may indicate low interest in peers and other abnormal social interactions (ASD factor I).

Juvenile Play. Pairs of juvenile rodents are allowed to freely interact, and the number, duration, and types of play behaviors can be measured. (Bey & Jiang, 2014). Low levels of juvenile play and limited duration indicate low interest in peers and/or difficulty maintaining interaction (ASD factor I).

Partition Test. The test rodent explores one-half of a partitioned arena with a novel rodent on the other side of the barrier. The time spent near the partition is a measure of social approach. (Bey & Jiang, 2014). Little or no time spent near the partition indicates low interest in peers (ASD factor I).

b. Communication Behavior

Rodents engage in complex social communication. Rats and mice communicate via vocal, visual, and chemical signals. Such communication between rodents is operationalizable variables relevant to social communicative alterations in neurodevelopmental disorders. The content and quality of the communication can be difficult to determine but the quantity and form of alterations can be quantified through various techniques, for instance:

Adult Ultrasonic Vocalizations. The test male rodent is introduced to a reproductive setting or given access to a stimulus, i.e., estrus female or female urine, or an agonistic setting, i.e., another male rodent (such as in resident-intruder paradigm). The number, duration, and types of ultrasonic calls are recorded (Bey & Jiang, 2014). Fewer and shorter calls may indicate communication deficits (ASD factor II) and may also represent decreased sociability (ASD factor I). Ultrasonic vocalizations can also be measured in females.

Pup Ultrasonic Vocalizations. Young rodents vocalize when away from their mothers. The quality and quantity of these vocalizations can be an important variable. The test rodent pup is briefly separated from its dam and littermates. The number, duration, and types of ultrasonic isolation calls are recorded (Bey & Jiang, 2014). Fewer and shorter calls may indicate communication deficits (ASD factor II) and may also represent decreased sociability (ASD factor I). Increased isolation induced calls can also indicate enhanced alarm signaling and perturbed distress perception in pups that could have relevance to neurodevelopmental impairments perhaps other than ASD.

Scent-Marking. Male rodents place territorial scent marks from multiple anatomic scant glands including facial glands and preputial glands. The test male rodent is exposed to urine from estrus females. The amount and proximity of male urine scent-marks to female urine stimuli is a measure of scent-marking communication behavior (Bey & Jiang, 2014). The number of scent

marks close to stimuli of opposite or same sex may measure olfactory communication (ASD factor II) and/or social interest (ASD factor I) (citations for this from my PhD lab and others e.g., Arakawa et al., 2007; Arakawa et al., 2008; Wohr et al., 2011).

c. Repetitive/Restricted Behavior

A hallmark of ASD is restricted repetitive motor behaviors (also colloquially called "stimming" in humans) and circumscribed interests. Self-directed behaviors and restricted and repetitive motor behaviors are measured rather easily in rodents through the tests discussed below and used in several of the studies reviewed here.

Holeboard Exploration. The test rodent explores an arena with a grid pattern of circular holes in the floor allowing for head-dips into the holes. The number and pattern of holes explored is a measure of perseverative exploration (Bey & Jiang, 2014). Perseverative exploration of only one of the holes represents restricted interests and repetitive behavior (ASD factor III).

Marble Burying. The test rodent is introduced to a cage containing marbles atop bedding. The number of marbles buried is a measure of compulsive digging and bedding sifting behavior (Bey & Jiang, 2014). The number of marbles buried are used to represent repetitive, compulsive-like behavior in rodents (ASD factor III).

Maze Reversal Learning. The test rodent must learn a new location of a hidden platform or escape hole in spatial mazes (see below). Its ability to learn a new location is a measure of cognitive flexibility (Bey & Jiang, 2014). An inability to learn the new location represents repetitive behavior and an insistence on sameness (ASD factor III).

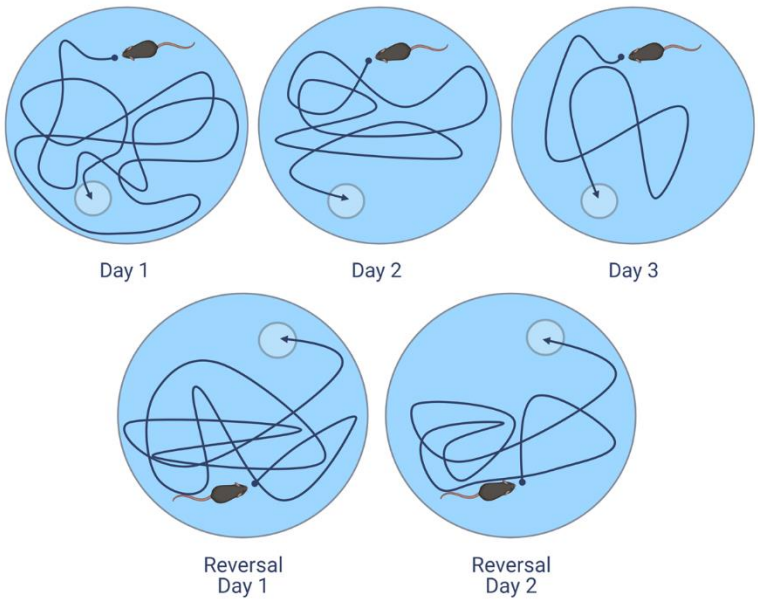


Figure 20. Morris Water Maze & Reversal Learning. Created with Biorender.

Nest Building. The test rodent is given pressed cotton material. The amount of material the rodent shredded and arranged into nest is a measure of nest construction ability (Bey & Jiang, 2014). Shredding (measured relative to weight of intact material) may be representative of repetitive, compulsive-like behavior (ASD factor III).

Repetitive Grooming. The test rodent is observed for self-grooming in its home cage or novel environments. The duration and bouts of grooming are measures of repetitive behavior, while skin lesions are measures of self-injurious behavior (Bey & Jiang, 2014). Grooming and lesions are used to measure repetitive behavior (ASD factor III). The pattern of grooming is highly stereotypical in rodents. In mouse models of ASD, that pattern of grooming can be even more rigidly stereotyped (Pearson et al., 2010).

B. ANIMAL MODELS OF ADHD

Rodent models of ADHD generally attempt to recapitulate attention problems and activity problems that people experience using conditioned and unconditioned behavioral tasks. Spontaneously hypertensive rats exhibit ADHD-like features including inattention and hyperactivity (Sagvolen et al., 2005). Genetic manipulations or dopaminergic brain lesions can also create features of ADHD in rodents.

Animal models of ADHD are somewhat less developed than animal models of ASD, and they have been criticized as not identical to ADHD in humans. However, they remain an essential tool in understanding ADHD behaviors and symptoms in humans, including factors that may ameliorate or worsen those symptoms (Russell, 2005). ADHD models in animals, like ASD models in animals, are not meant to be exact duplicates of these quintessentially human disorders. Instead, scientists seek to better understand symptoms of ADHD which can be readily modeled in animals, including inattention, impulsivity, and hyperactivity.

Genetic manipulation and selective breeding have resulted in several rodent species that are genetic models of ADHD-like phenotypes, including the spontaneously hypertensive rat (SHR) and dopamine transporter knock-out (Dat KO) mouse. SHR models demonstrate face validity in that they are hyperactive, impulsive, and inattentive; they demonstrate construct validity in that they exhibit neuropathological changes similar to those observed in humans with ADHD; and they demonstrate predictive validity in that common ADHD treatments (i.e., psychostimulants and guanfacine) attenuate their hyperactive, impulsive, and inattentive symptoms (Regan et al., 2022). Dat KO (dopamine transporter knockout) mouse models demonstrate face validity in that they are hyperactive, impulsive, and inattentive; they demonstrate construct validity in that Dat KO mice exhibit reduced dopamine reuptake and ADHD in humans is associated with dopamine signaling alterations; and they demonstrate predictive validity in that ADHD treatments, attenuate their hyperactive symptomology (Regan et al., 2022). Variants of this species, including Dat KO heterozygous (Dat KO hets) and DAT Knockdown (Dat KD) are also used to model ADHD (Regan et al., 2022).

Other genetic models of ADHD include the Snap-25 KO coloboma mouse, dopamine receptor KO mice, guanylyl cyclase-C KO mice, and latrophilin-3 KO mice and rats (Regan et al., 2022). ADHD symptoms can be modeled in these rodent species through the tests below, many of which are also used in several of the studies reviewed in this report.

1. Animal Behavioral Testing and ADHD

a. Hyperactivity

Hyperactivity in animal models is reflected by increased or changed activity during some or all of the activity test or habituation test in an arena or novel cage. Common tests indicating hyperactivity include:

Light/Dark Exploration Box. The test rodent is confined to a dark zone in a box. The rodent is then transferred to a light zone and allowed to commute freely between the two zones, as depicted in Figure 21. Its behavior during the initial confinement and after the transfer is recorded and scored. Unusually high motor activity is treated as indicating hyperactivity associated with ADHD. The light/dark exploration task is generally a test used for evaluating exploration and risk assessment, fear, and anxiety-related behavior of a rodent but it can be utilized for this application as well.

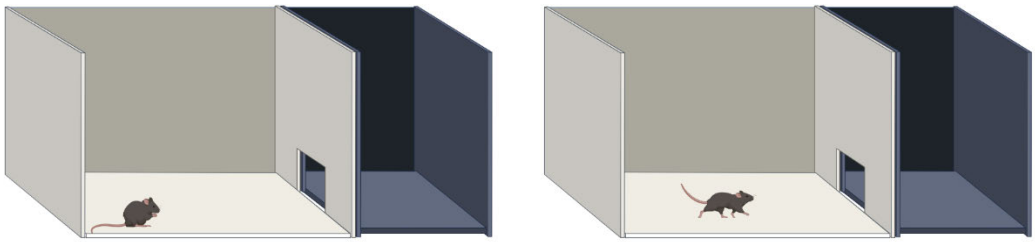


Figure 21. Light/Dark Exploration Box.
Created with Biorender.

Open Field Test. The test rodent is placed in the center or near the walls of the test apparatus for the duration of the test (usually 5 minutes, but up to 60 minutes or more) and target behaviors are measured, including but not limited to horizontal locomotion, rearing or leaning ("vertical activity"), grooming, distance traveled, time spent moving vs. still, and time spent in central zone(s) vs. peripheral zones. Increased activity in the open field apparatus may indicate hyperactivity which is linked with ADHD in humans. A variant of this test is with a stimulant given just before the test to evaluate how sensitive a model is to stimulant-induced hyperlocomotion.

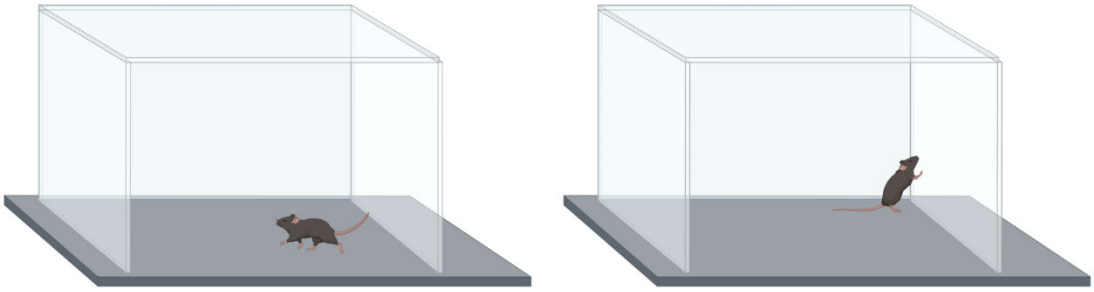


Figure 22. Open Field Test.
Created with Biorender.

Voluntary Wheel-Running Activity. The test rodent is placed in a cage with an attached running wheel. Wheel rotations, running speed, and duration of running are measured as indicators of hyperactivity. This measure can also reveal disruptions to diurnal cycles.

Y-maze. The test rodent is placed in a Y-shaped maze that has three equally-sized radial arms with raised walls and is allowed to freely explore the maze when the test begins. The total number of arm entries and consecutive entries into each arm without revisiting previously-entered arm(s) (known as "spontaneous alternation") are measured. An increased number of arm entries indicates hyperactivity (ADHD factor II) and reduced spontaneous alternation indicates spatial memory deficits, which have been recognized in animal models of ASD and ADHD though it is not a core criterion for either disorder.

b. Impulsivity

Animal models measuring impulsivity, i.e., premature responding and recorded as bursts of responses with short inter-response times, look at the number of responses with short inter-response times to determine the degree of impulsiveness. Common tests to model impulsivity include:

Cliff-Avoidance Reflex (CAR) Test. The test rodent is placed on an elevated platform supported on a rod (similar to a barstool). The rodent's behavior is recorded. The length of time to first fall is calculated as a measure of impulsivity associated with ADHD.

Stop-Signal Reaction Time (SSRT) Test. The test rodent is placed in a chamber and trained to poke first a left lever and then a right lever to secure a reward. But, in 20% of cases, the test rodent receives a stop-signal tone upon pressing the left lever—failure to stop is punished. SSRT measures inhibition of a response that has already been initiated (i.e., the ability to stop). The inability to stop is attributed to impulsivity, and thus associated with ADHD.

T-Maze Test. The test rodent is placed in a T-maze and trained to choose between a small immediate reward and a larger reward that is delayed 30 seconds. Choosing the smaller, but immediate, reward is tied to impulsivity associated with ADHD.

c. Attention

ADHD is associated with problems with attention. In rodents, attention can be evaluated by training animals to respond to cues with varying intensities that require sustained attention. An example is:

5-Choice Serial Reaction Time Task (**5CSRTT**). As described in my group's study (Baker et al., 2023), mice are trained to watch a touchscreen where one of 5 squares will light up for a brief duration. If they touch the light, they get a food reward. The mice are food restricted so they are motivated to work for the food reward. By measuring how many trials it takes the mice to learn the task one can measure learning. Then, we reduce the duration that the light stays on all the way from 32 seconds to less than one second. Animals that have problems maintaining attention will miss trials and would show fewer correct trials or show more missed (omitted) trials. The task can be made even more challenging by adding distracters or adding punishment if the mice make

a mistake. Also, the number of premature choices that a mouse makes can be indicative of impulsive characteristics.

C. The Role of Animal Model Data in Identifying Causes of Neurodevelopmental Disorders

Animal models of neurodevelopmental disorders like those described above provide critical information to researchers investigating the relationship between environmental exposures and these uniquely human conditions. The developmental neurotoxicity of chemicals and medications can be studied by exposing rodents to the test substance during critical periods of neurodevelopment and measuring the extent to which resulting the behavioral, structural, and/or chemical alterations resemble those found in animal models of NDDs.

Animal studies also allow for the investigation of dose-response relationships between exposures and NDD-like outcomes. By exposing animals to different doses of a chemical or medication, researchers can determine whether there is a dose-dependent relationship between exposure levels and the severity of behavioral, structural, or chemical alterations. This information is helpful for assessing the causative relationship between an exposure and neurodevelopmental outcomes and is key to establishing exposure guidelines. Absence of dose response information (i.e., single dose studies) can nevertheless provide causal and informative information for causal preclinical evidence.

Investigating how developmental exposure to chemicals and medications impact brain structure, neurotransmitter systems, and gene expression in animal testing provides valuable insights into potential pathways and mechanisms through which environmental exposures cause NDDs. The FDA's review produced in this case cites the importance of animal studies to addressing the safety of APAP, noting that it would be "unlikely that further observational studies will provide more clarity without more mechanistic data." While animal studies are not a perfect predictor of pharmaceutical safety in humans, they offer an important tool to examine real-world effects of a compound in a living subject. The makers of Tylenol actively use animal models in their preclinical drug development testing and rely upon results from animal testing (see, e.g., Goethals, 2023).

Animal studies also allow researchers to exert significant control over experimental conditions (e.g., genetics, environment, and exposure). As a result, important variables such as the chemical or medication exposure can be isolated and studied without confounding influences. For example, confounding due to genetic variability can be controlled by experimenting on genetically homogenous subjects (i.e., inbred rodent lines). Laboratory animal experiments can control for the time of day of drug administration and the dosage(s). They normalize other environmental factors such as dietary differences, sexual experience, pathogen burdens, birth order effects, and various other climate, health, housing, psychological and other potential confounding variables.

To illustrate how we can compare species, APAP doses, and evidence of neurotoxicity across humans and mice, I plotted a simple summary of human and mouse oral APAP toxicity. Figure 23 shows oral therapeutic doses for analgesia or antipyresis (in green), doses that could be mildly or moderately hepatotoxic but in a range that the subject can recover (in yellow), and doses that would be lethal without intervention (in orange/red). I then overlayed (in purple) the range of

doses administered to mice in published literature that resulted in neurotoxicological outcomes, whether they be neurotransmission, behavioral changes, relevant transcriptional perturbations or the like (listed below). What is clear is that there are effects at sub-lethal and sub-hepatotoxic doses of APAP.

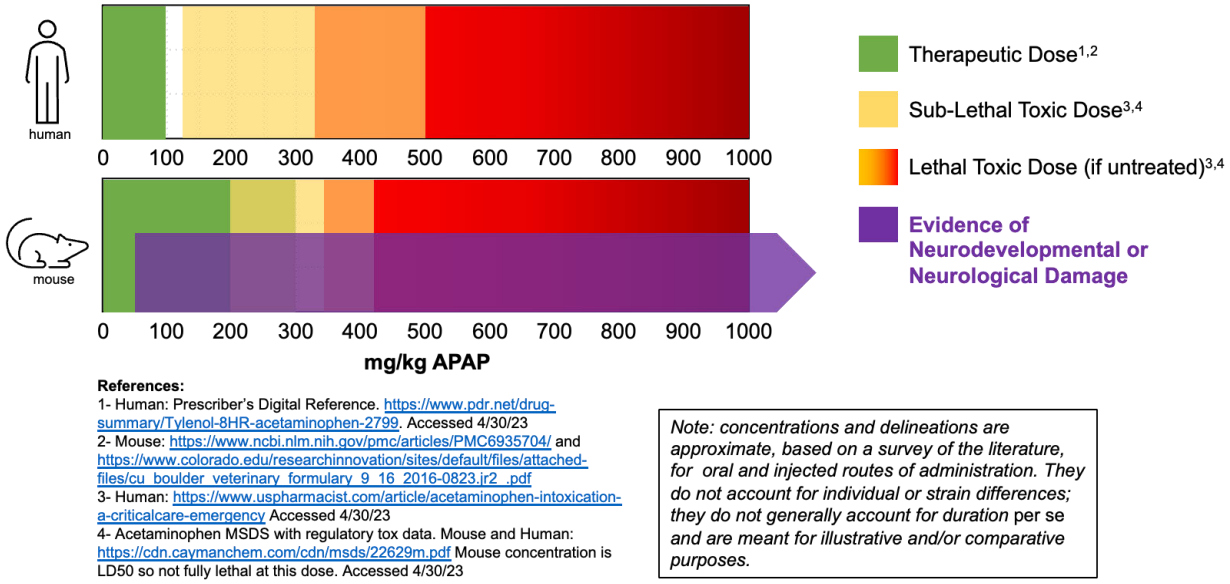

human
0 100 200 300 400 500 600 700 800 900 1000
mouse
0 100 200 300 400 500 600 700 800 900 1000
mg/kg APAP
Therapeutic Dose1,2
Sub-Lethal Toxic Dose3,4
Lethal Toxic Dose (if untreated)3,4
Evidence of Neurodevelopmental or Neurological Damage
References:
1- Human: Prescriber's Digital Reference. https://www.pdr.net/drug-summary/Tylenol-8HR-acetaminophen-2799. Accessed 4/30/23
2- Mouse: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6935704/ and https://www.colorado.edu/researchinnovation/sites/default/files/attached-files/cu_boulder_veterinary_formulary_9_16_2016-0823.jr2_.pdf
3- Human: https://www.uspharmacist.com/article/acetaminophen-intoxication-a-criticalcare-emergency Accessed 4/30/23
4- Acetaminophen MSDS with regulatory tox data. Mouse and Human: https://cdn.caymanchem.com/cdn/msds/22629m.pdf Mouse concentration is LD50 so not fully lethal at this dose. Accessed 4/30/23
Note: concentrations and delineations are approximate, based on a survey of the literature, for oral and injected routes of administration. They do not account for individual or strain differences; they do not generally account for duration per se and are meant for illustrative and/or comparative purposes.

Figure 23. Created for purposes of this report.

At a high level, both mice and rats are essential to preclinical models. Laboratory rodents are ideal for developmental neurotoxicity studies because their reproduction, gestation, and lactation are remarkably similar to humans. Rodents reproduce and develop at a faster rate than larger animals like primates, and because they are smaller, scientists are able to design more, larger and more robust studies. They also engage in intensive parental care, communication, and other social behaviors much like their human counterparts. This is true despite the fact that the gestation and development period of rodents is substantially shorter than that of humans, as depicted in Figure 24, below. For example, gestation in rats is completed within 23 days of conception as compared to 280 days in humans. For mice, the period is approximately 19-19.5 days. With regard to neurodevelopment, post-natal days 3 to 10 in a mouse are equivalent to the third trimester of pregnancy in humans (Philippot et al., 2017). Understanding these differences, scientists can account for them in their study design. Additionally, while animal studies are not a 1:1 comparison to humans, they still provide important, relevant information that can be extrapolated to the human experience.

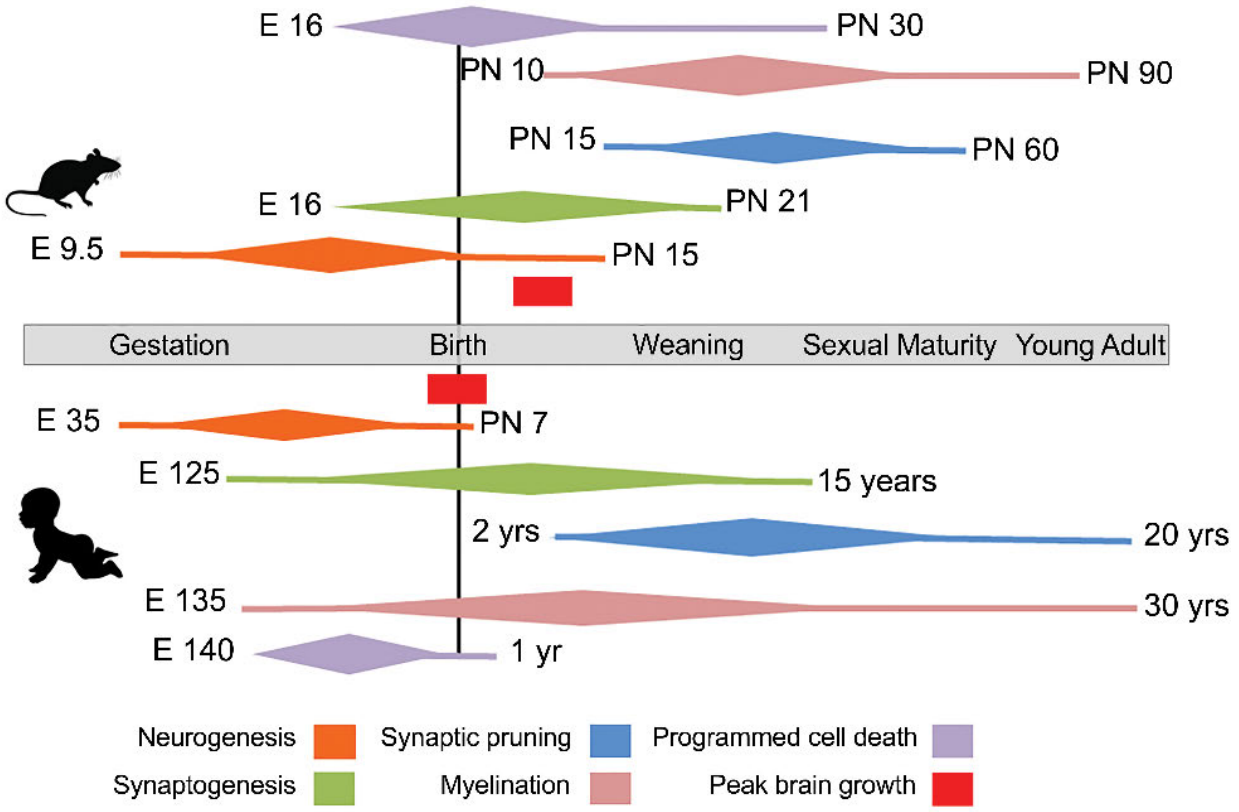


Figure 24. Comparison of brain development milestones of rats relative to humans. Approximate timelines of these processes are shown in relation to anchor events of birth, weaning, sexual maturity, and adulthood. Brain growth spurts are shown in red. Individual processes are color-coded, with peak activity indicated by the widest portion of the diamond. Rats and mice are broadly equivalent in these milestones. Reproduced from Zeiss, 2021.

Peak brain growth, neurogenesis, and programmed cell death that is complete or nearing completion at term in a human neonate persists into the first 2-3 postnatal weeks in a rodent (Pressler & Auvin, 2013). Accordingly, preclinical APAP neurotoxicology researchers may choose to include this 3rd trimester human brain period equivalent in their translational exposure design.

For example, in Philippot et al., 2017, groups of mice were administered either saline or two subcutaneous injections of 30 mg/kg APAP four hours apart on post-natal day (PND) 3, PND 10, or PND 19. Mice underwent behavioral testing at 2 months of age which corresponds to early adulthood in humans. The mice that were administered APAP on PND 3 and 10 showed significant changes in spontaneous behaviors compared to control mice, whereas those administered on PND 19 did not. Though administered to mice postnatally, the results are clinically relevant to a human fetus exposed to a therapeutic dose of APAP during the third trimester. The results of this study, which are discussed in more detail later in this report, show that APAP can perturb neurodevelopment during multiple critical stages, and is most damaging prenatally as opposed to postnatally.

In addition, animal study designs allow researchers to explore a molecule's effect on genes and gene expression. As discussed in more detail below, current research techniques allow scientists to examine epigenetic changes in response to toxic substances. Changes to the epigenome can alter gene expression and can help us understand complex neurodevelopmental outcomes. One way to monitor epigenetic changes is to examine DNA methylation. DNA methylation is a chemical modification that can silence or regulate the expression of genes. DNA methylation is affected by factors including exposures to environmental toxins and medications. DNA methylation analysis can allow us to identify specific genes affected by a chemical. Histone modification offers another method by which we can examine the effect of environmental exposures on gene expression. Histone proteins are used to package DNA into chromosomes. Modification to histones, like modifications in DNA methylations, can alter gene expression. Histology and microscopy techniques, including the use of immunomarkers,[6] in immunohistochemical techniques also allow us to examine evidence of changes in protein levels, structural changes in tissue, DNA and other molecular damage, cell composition changes, and even cell death. Histological and staining approaches can be used to evaluate the size of structures and cells as well as their abundance and arrangement. These techniques are like what is often done by pathologists evaluating human tissue to diagnose disease or determine causes of death and disability.

Animal study designs can also use comprehensive molecular "omics" tools such as genomics, transcriptomics, metabolomics, methylomics, or proteomics. [Adding "omics" to the end of a term simply means that the researcher is capturing the entirety of something]. Omics techniques are one of the most powerful tools at our disposal to understand the effect of toxicants on complex health outcomes. Omics refers to a collection of high-throughput techniques that allow researchers to perform comprehensive analysis of molecules and molecular processes, including genes, proteins, and metabolites. Omics analyses allow researchers to better understand biological processes and ways they can be disturbed on both a larger and more detailed scale (Figure 25, below). In my research group's study, addressed in more detail later in this report, we used transcriptomics techniques to show the effect of APAP on a multitude of specific genes.

[6] An immunomarker, also referred to as an immunohistochemical marker or immunohistochemistry (IHC) marker, is a substance or molecule employed to identify and visualize particular proteins or antigens within cells or tissues. These immunomarkers find extensive application in research, diagnostics, and pathology for the identification and characterization of cellular components, exploration of disease mechanisms, and assistance in diagnosing and classifying various conditions.

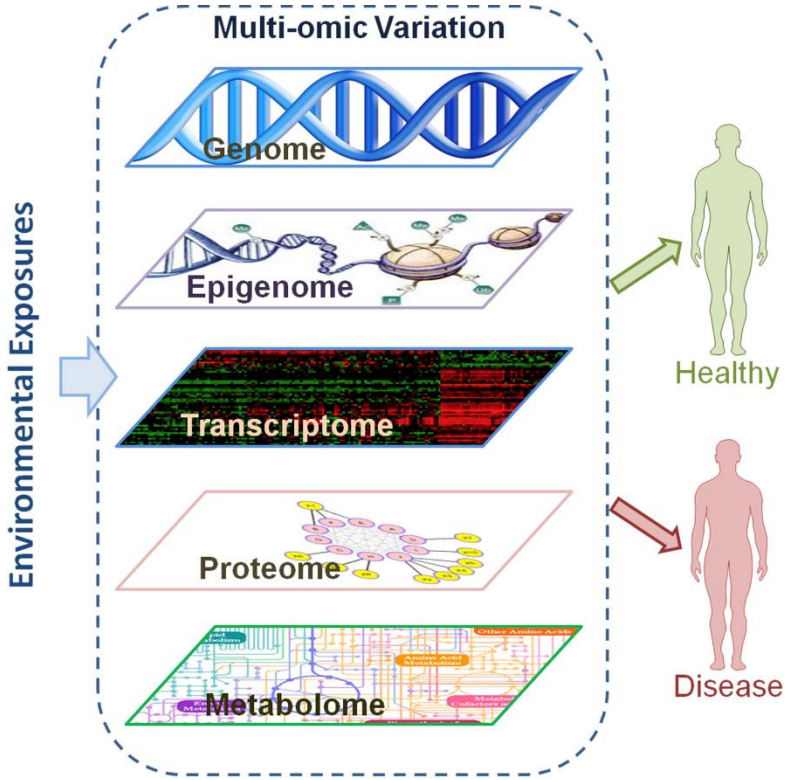


Figure 25. Levels of "omics" analyses. At the top level is the genome, etc, to metabolome which is the comprehensive evaluation of all metabolites in a biospecimen. Modern systems biology tools integrate across these levels of analysis to understand exposures (left) to health and disease states (right). This concept is critical to understanding causal roles of prenatal APAP exposure and neurodevelopmental disorder outcomes. The weight of evidence should rely substantially on untargeted omics techniques.
Reproduced from Sun & Hu, 2016.

Analytical techniques are often necessary to measure biomolecules. Proteins and hormones can be quantified using Western Blot or Enzyme-linked immunosorbent assay (ELISA). HPLC-EC assay refers to a specific analytical method that combines High-Performance Liquid Chromatography (HPLC) with Electrochemical (EC) detection. It is commonly used to quantify and analyze compounds that can undergo electrochemical reactions, such as neurotransmitters, catecholamines, or other electroactive substances. Published studies list the specific analytical tools used and how they are validated. Best practices are agreed upon in the disciplines of study and most academic journals have standards of reporting for the validation and quality standards for such techniques.

X. APAP'S MECHANISMS OF NEURODEVELOPMENTAL INJURY

Understanding the mechanisms by which prenatal APAP exposure leads to neurodevelopmental disorders in offspring helps understand the causal relationship between prenatal APAP use and the symptoms of neurodevelopmental disorders, including ASD and ADHD. The studies analyzed and discussed in this report reveal multiple complex and intermingled mechanisms of action. My discussion below focuses on the mechanisms of action that are most central to the impact of APAP on human neurodevelopment from my perspective as a neurotoxicologist.

A. APAP Causes Formation of a Toxic Metabolite, Inducing Oxidative Stress and Mitochondrial Inhibition

In my opinion, based on my experience studying APAP and my weighing of the preclinical data as discussed below, the primary mechanism by which APAP causes neurodevelopmental disorders is through oxidative stress caused by the creation of a reactive metabolite NAPQI and the depletion of glutathione (GSH). Oxidative stress refers to a state of imbalance between the production of reactive oxygen species (ROS) and the capacity of an organism to detoxify and repair the damage induced by these species. ROS are highly reactive molecules that can cause damage to cellular components including proteins, lipids, and DNA. While ROS are naturally produced as byproducts of normal cellular metabolism, excessive amounts can lead to cellular damage and dysfunction. That dysfunction may manifest as DNA damage, cell death, and dysfunction. During brain development, these processes can cause disruptions in neurodevelopment leading to outcomes including symptoms of ASD and ADHD.

1. Oxidative Stress

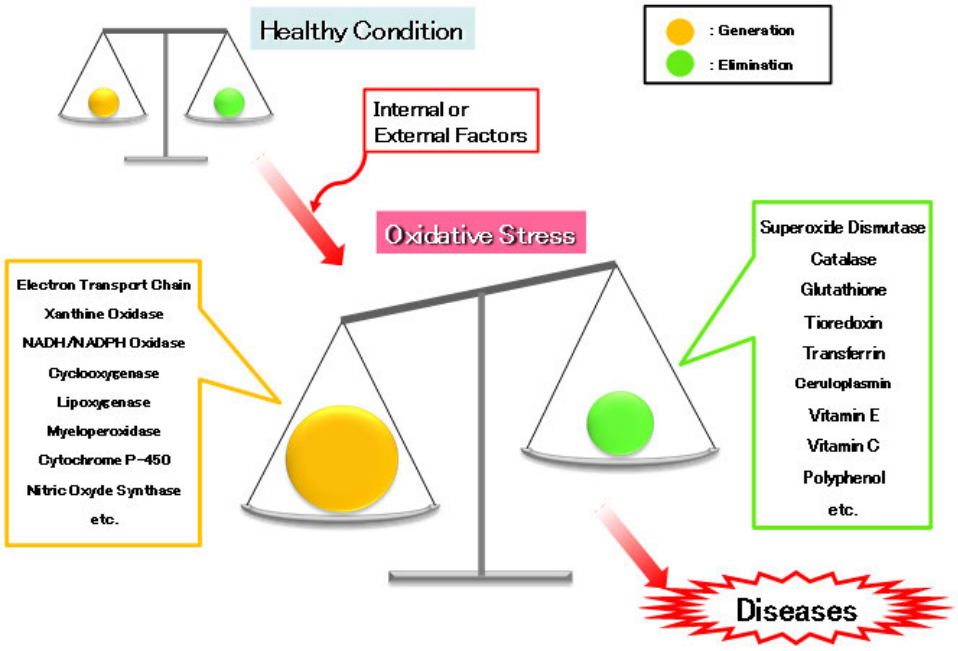


Figure 26. Graphic depicting oxidative stress as an imbalance of free radicals and antioxidant and detoxification systems. Oxidative stress is depicted as a left leaning imbalance via free radical sources outweighs antioxidant capacity. Reproduced from https://www.graphyonline.com/archives/IJPNC/2015/IJPNC-103/index.php?page=figure&num=1.

Oxidative stress refers to the imbalance of pro-oxidants (free radicals such as reactive oxygen and reactive nitrogen species) and antioxidants. The figure above provides an overview of how cells and systems maintain a balance of free radicals that are formed from metabolic systems such as the mitochondrial electron transport chain that are balanced by the presence of antioxidant and detoxification systems. It is well established that APAP causes oxidative stress. As discussed previously in this report, APAP is rapidly metabolized upon ingestion through a series of enzymatic reactions. One of the primary metabolic pathways involves the formation of a toxic

metabolite, NAPQI, through the action of the cytochrome P450 enzymes (specifically the enzyme cytochrome P450 2E1, also called CYP2E1). Importantly, CYP2E1 is expressed in the prenatal brain (Brzezinkski et al., 1999) including as early as 2nd trimester with higher levels of expression in the 3rd (O'Hara et al., 2015).

Under normal circumstances, NAPQI is rapidly detoxified by reduced glutathione (GSH). GSH is an important antioxidant molecule present in cells throughout the body that plays a crucial role in mitigating oxidative stress and maintaining cellular redox balance. During periods of increased oxidative stress, the demand for GSH increases as it is utilized to neutralize ROS and counteract the damaging effects. However, prolonged or excessive oxidative stress can deplete GSH levels, leading to a compromised antioxidant defense system. If the level of APAP exceeds the liver's GSH reserves, NAPQI can accumulate, leading to oxidative stress. Without sufficient GSH reserves, the body is left without a defense to the toxic effects of NAPQI.

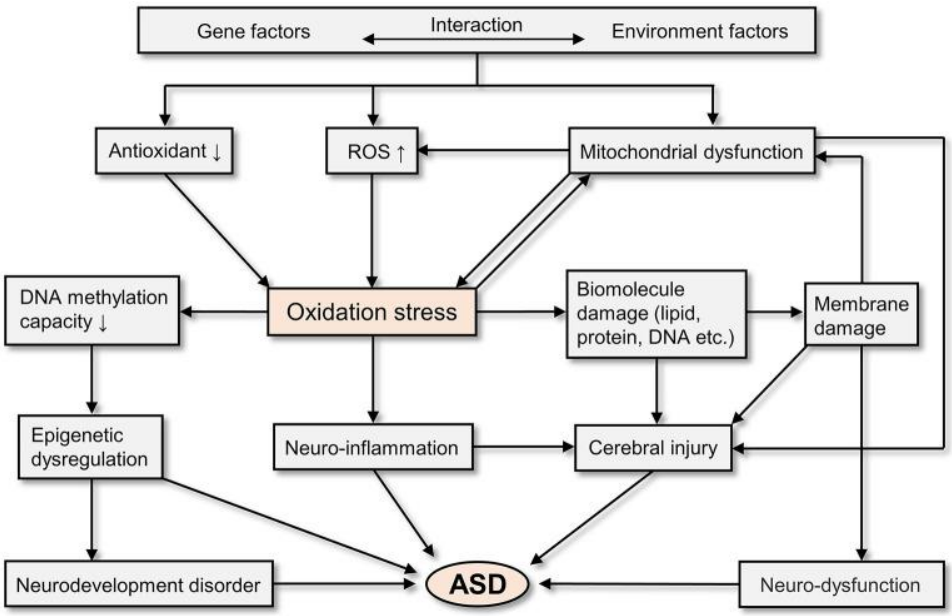


Figure 27. Graphical depiction of the ways oxidative stress can perturb neurodevelopment and lead to neurodevelopmental disorders.
Reproduced from Liu et al., 2022.

A number of techniques can be used to quantify and measure levels of oxidative stress caused by individual compounds, including in silico, in vitro, and in vivo studies. Even in human subjects, biomarkers can be used to demonstrate the level of oxidative stress caused by compounds including APAP. 8-hydroxy-2'-deoxyguanosine (8-OHdG) is a specific biomarker of oxidative stress used in both human and animal studies. In humans, higher concentrations of APAP have been shown to be associated with higher 8-OHdG in the umbilical cord (Anand et al., 2021). Higher urinary concentrations of APAP are also associated with higher levels of oxidative stress biomarkers including 8-OHdG (Li et al., 2022).

In the brain, oxidative stress can significantly impact neurodevelopment, particularly during the critical periods of early brain development. In general, the brain is particularly susceptible to oxidative stress due to its high metabolic rate and oxygen consumption. During

neurodevelopment, oxidative stress can result in damage to developing neurons and their supporting cells, leading to impaired brain function and cognitive deficits. Additionally, excessive ROS production can trigger inflammation and cause further damage to developing brain cells. Numerous studies have demonstrated a link between oxidative stress and neurodevelopmental disorders, including ASD and ADHD (Curpan et al., 2021; Hayashi et al., 2012; Liu et al., 2022; Nishimura et al., 2021; Usui et al., 2023; Wells et al., 2009). In individuals with these disorders, there is evidence of increased oxidative stress and decreased antioxidant defense systems in the brain, which may contribute to their development and progression. Other neurotoxic substances known to cause neurodevelopmental outcomes, including lead, also show evidence of causing oxidative stress on the developing brain (Nishimura et al., 2021).

Oxidative stress caused by APAP can also leave a developing brain vulnerable to other causes of oxidative stress including other toxic exposures. The reverse is also true. Individuals with low detoxification abilities because of, for example, lower GSH levels, could show amplified toxicity in response to APAP, show longer persistence of NAPQI radicals and acquire more damage from oxidative stress. This is particularly relevant here, where research suggests that humans have less GSH when pregnant than when non-pregnant (Balasubramanian & Birundha, 2019). Accordingly, pregnant people may have less ability to detoxify NAPQI which could cause maternal cellular damage which could cause inflammatory responses that signal damage to the fetal brain development.

As discussed previously in this report, CYP2E1 is expressed at various times in various locations in the brain. The varying locations of CYP2E1 expression in the fetal brain over the course of development explains the heterogeneity of outcomes in APAP-exposed animals and humans. Because the brain changes every day during in utero development, small changes in terms of the timing of exposure could lead to different parts of the brain being exposed to NAPQI as CYP2E1 expression changes dynamically over time.

2. Mitochondrial Damage

APAP's toxic metabolite, NAPQI, can also damage mitochondria, the organelles which produce adenosine triphosphate (ATP), the main source of energy in cells. Normal mitochondrial function is critical for normal brain development, in part because the brain is a very energy-intensive organ and requires a great deal of ATP to function normally.

Oxidative stress can damage mitochondria. Mitochondria also have their own DNA, separate from the nuclear DNA present in the cell, and oxidative stress can damage mitochondrial DNA, leading to mutations and impaired function. Mitochondrial DNA, also called mtDNA, is particularly vulnerable to oxidative damage because it is located near the site of ROS production during oxidative phosphorylation. Oxidative stress can cause mtDNA mutations, deletions, and strand breaks, leading to impaired mitochondrial function and energy production. Oxidative stress can also dysregulate proteins involved in mitochondrial processes, affecting their structure and function. In addition, oxidative stress can lead to lipid peroxidation, which can disrupt mitochondrial membranes, and compromise their integrity. Dysregulation of calcium levels within mitochondria can occur, further affecting mitochondrial function.

Once bound to the proteins in mitochondria, NAPQI can result in decreased ATP, leading to a cascade of consequences ranging from additional formation of reactive oxygen nitrogen species, altered gene expression, and apoptosis.

3. Oxidative Stress and Inflammation

In addition to its own effects, oxidative stress also has a complex and bi-directional relationship to inflammation. When neuroinflammation (inflammation of the brain) occurs, immune cells are activated, and a release of pro-inflammatory molecules occurs. Oxidative stress can promote neuroinflammation by activating signaling pathways that induce the production of pro-inflammatory molecules, such as cytokines, chemokines, and reactive oxygen species. These inflammatory mediators can further perpetuate oxidative stress. Accordingly, inflammation and oxidative stress do not act alone. Given the early life neuroinflammatory etiology of ASD and ADHD, any stressor that can cause oxidative stress and/or inflammatory signaling has the potential to trigger the cellular and synaptic changes that underlie ADHD.

4. Toxic and Therapeutic Properties of APAP are Contextual

APAP can show neuroprotection and anti-inflammatory influences when used alongside other agents or manipulations. APAP might also exert helpful effects at small doses, such as improvements in learning or reductions in anxiety at low concentrations. However, it is critical that those effects be viewed in context of potentials for disturbances to developmental programs during gestational brain development. In the context of existing damage or stress, APAP can also act therapeutically to remove pain and reduce the propagation of damage in specific contexts. However, APAP is also capable as acting as the trigger of inflammation and oxidative stress. When APAP is administered at lower doses and durations to an animal in a disease state, its effects can activate the body's ability to produce antioxidants. However, that protective effect can eventually give way to the inflammation, oxidative stress and other toxic processes caused by APAP. These considerations are important insofar as arguments to the safety of APAP to the developing brain cannot be derived from studies showing that APAP improves outcomes when there is a major stressor or insult, such as studies where there is a brain lesion (e.g., APAP improves brain recovery when there is an ibotenic acid lesion i.e., Leroux et al., (2010)). In other words, although APAP is therapeutic under certain conditions of damage in the nervous system, it does not mean it does not cause harm in other health or disease states.

B. DNA Damage, Epigenetic Changes and Cell Death

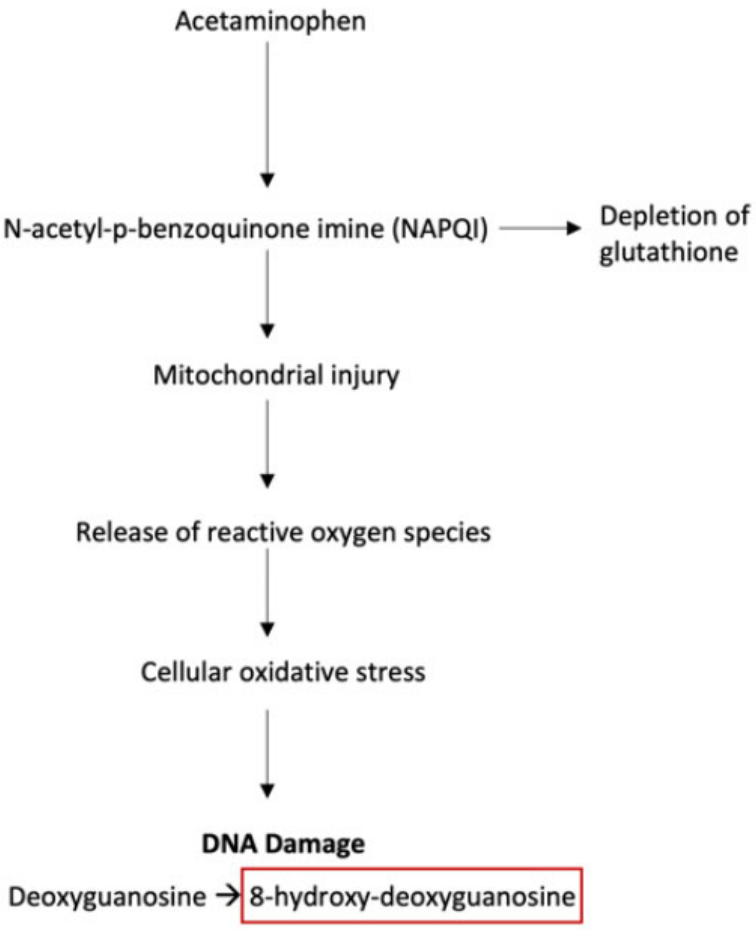


Figure 28. Pathways from APAP exposure to DNA damage. Reproduced from Anand et al., 2021.

1. Cellular Stress Effects on Neuronal Function and Neurodevelopment

Oxidative stress and inflammation lead to reduction in synaptic gene expression and can lead to alterations in cell migration, stem cell and progenitor pool maintenance and differentiation, and changes to neuronal expression of specialized neuronal gene programs. Neurons are particularly avoidant to programmed cell death (Kole et al., 2013), in part, because they are cell types that cannot replace themselves. In my ten years of studying gene transcriptional responses of nervous system cells and tissues to stressors such as drugs and environmental chemicals, my colleagues and I have noted that neurons and brains will downregulate genes that are specialized to neurons, such as synaptic genes, and genes that are long (King et al., 2013; Zylka et al., 2016). Most long genes happen to be synaptic genes. By downregulating long synaptic genes, neurons can protect themselves from damage associated with oxidative stress and instead direct limited energy towards transcriptional programs to detoxifying and antioxidant responses (Pearson et al., 2016). APAP causes pronounced oxidative stress and mitochondrial inhibition as described in the section above. This would lead to the expression signature as shown in the figure below, in cluster

2 which shows the effect of mitochondrial electron transport chain inhibitors which downregulated synaptic genes at the bottom and upregulated immune/inflammatory genes and endogenous antioxidant genes such as NRF2 target genes. The importance of this in the context of neurodevelopment cannot be overstated. Loss of function of any one of these long neuronal genes (which also include nuclear envelope proteins, chromatin remodelers, transcription factors, etc.) can cause ASD or other neurodevelopmental disorders. The downregulation of dozens of them even for a very brief time at critical points of synaptogenesis, neuronal migration, experience dependent circuit maturation, or cell proliferation could be a central integrating mechanism by which a pro-oxidant, mitochondrial poisoning drug could cause a neurodevelopmental disorder such as ASD or ADHD.

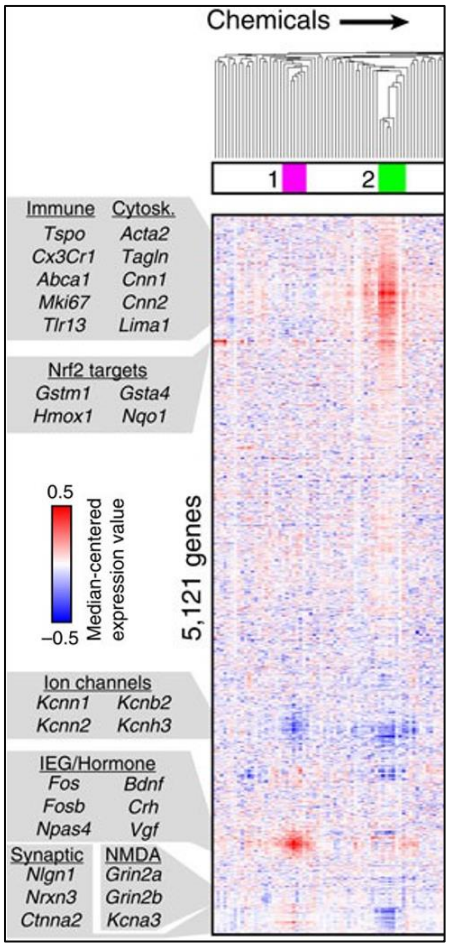


Figure 29. Green box and entire column below it depicts "cluster 2" mitochondrial inhibitors which cause high levels of oxidative stress. Those chemicals cause the upregulation of inflammatory and oxidative stress genes such as Cx3Cr1. Concomitantly, they lead to the downregulation of ASD genes such as synaptic genes including NLGN1 and glutamate receptor subunits such as Grin2b. The concentration of the mitochondrial toxicants used in this study did not kill the cells. These are sub-cytotoxic doses, so this demonstrated that the neurons were downregulating the genes that allow them to perform their specialized neuronal functions such as proper electrochemical communication, to respond to and detoxify the pro-oxidant exposures.
Modified from Pearson et al., 2016.

2. DNA Damage

As part of the cascade of events caused by exposure to NAPQI, DNA damage can occur. Once created by APAP metabolism, NAPQI is capable of causing damage to DNA molecules by inhibiting enzymes that prevent DNA from becoming tangled (Bender et al., 2004) and by creating oxygen free radicals that can lead to DNA damage. DNA damage refers to any alteration or modification in the structure of the DNA molecule. DNA, or deoxyribonucleic acid, is the genetic material that carries the instructions for the development, functioning, and reproduction of all living organisms, including the human brain. DNA contains the blueprint for protein synthesis, which is essential for cellular structure, function, and regulation. Damage to the DNA molecule can include breaks, chemical modifications, and cross-linking between DNA strands. It is well established that toxic exposures can cause DNA damage. And while our bodies are generally adept at repairing DNA damage, some damage may remain unaddressed or undergo incomplete or incorrect repair, resulting in permanent modifications to the DNA sequence.

NAPQI may form adducts with DNA which can lead to mutation upon repair (Li et al., 2019). More common is a scenario in which reactive oxygen species production from mitochondrial inhibition leads to the formation of 8-hydroxy-2'-deoxyguanosine (8-oxo-dG) which through its repair, can lead to mutations. Taken together, while APAP may not show remarkable carcinogenic properties, it is a mutagen capable of causing damage to various molecules including DNA. DNA mutation is closely tied to neurodevelopmental disorders. Some neurodevelopmental syndromes are caused by specific, highly penetrant DNA mutations such as Fragile X Syndrome, whereas some DNA mutations may not result in dysfunction. However, other neurodevelopmental disorders like ASD and ADHD can be caused by multiple mutations across many genes, increasing the likelihood of neurodevelopmental disfunction. In fact, emerging studies suggest that there are patches of mosaic mutations in ASD (Rodin et al., 2021) and ADHD (Poliquin et al., 2021) that could be caused by chemical exposures in utero.

The consequences of DNA damage can be far-reaching because of the possibility that DNA damage disrupts gene function and associated processes. The genetic sequences within DNA provide instructions for the synthesis of specific proteins, and any alterations to these sequences can lead to errors or abnormalities during protein production. Negative effects from DNA damage can include genetic mutations, impaired cellular function, hastened aging, and increased susceptibility to cancer. In the context of neurodevelopment, DNA damage can cause neurodevelopmental disorders.

The brain is particularly sensitive to the results of DNA damage, in part because the brain has high metabolic activity. When DNA damage occurs in the brain, it can have a significant effect on the structure and function of brain cells and can lead to neurological and neurodevelopmental disorders. Since up to half of the cells of the brain are neurons that do not undergo replication (mitosis), they also show limited DNA repair capacity through replicative DNA repair machinery as well as a limited capacity to undergo programmed cell death and cell replacement. DNA damage has been implicated in the development and progression of neurodegenerative diseases like ALS, Parkinson's, and Huntington's disease (Madabhushi et al., 2014). Some of this DNA damage underlies somatic mutations in subsets of cells which are linked to adult-onset neurological diseases as well as those associated with insults in utero such as ASD. In fact, ASD is associated

with higher rates of somatic mosaic mutations in the brain indicating that early life DNA damaging insults could contribute to ASD via this somatic mosaic mutational process (Baker et al., 2021).

More importantly in terms of APAP and neurodevelopment, DNA damage can lead to altered gene expression. In the brain, genes regulate the production of proteins that are crucial for neuronal development, synaptic plasticity, and overall brain function. As previously discussed in this report, normal gene expression is particularly crucial during early brain development as the brain is rapidly growing and changing. DNA damage can affect the expression of genes in brain cells, thereby altering the synthesis of abnormal proteins or protein functions. These alterations disrupt the delicate balance of molecular processes in the brain that occur, particularly in a developing fetus, and can lead to neurological dysfunction.

When DNA damage is severe enough, it can also cause programmed cell death, otherwise known as apoptosis. As discussed above, individual cells have sophisticated mechanisms to sense and attempt to repair DNA damage. When the DNA damage is overwhelming and cannot be repaired, the cell actives tightly regulated and complex processes to protect the integrity of the organism through programmed cell death.

3. Cell Death

APAP has also been shown to cause apoptosis in brain cells as demonstrated, for example in a study examining brain cells in rats (Posadas et al., 2010). Apoptosis, or programmed cell death, can be a highly undesirable outcome during neurodevelopment and has potentially major consequences in terms of the disruption of normal brain development. While some amount of apoptosis during neurodevelopment is normal and necessary, disturbances in its regulation and excessive apoptosis can have negative consequences. Excessive apoptosis can lead to the loss of essential neurons and disruption of neural circuits. The loss of these neurons can impair various cognitive, sensory, and motor processes. Apoptosis can also affect synaptic connections, disrupting the extensive connections between neurons and leading to imbalanced or abnormal circuitry which can impair information processing, learning, and memory formation.

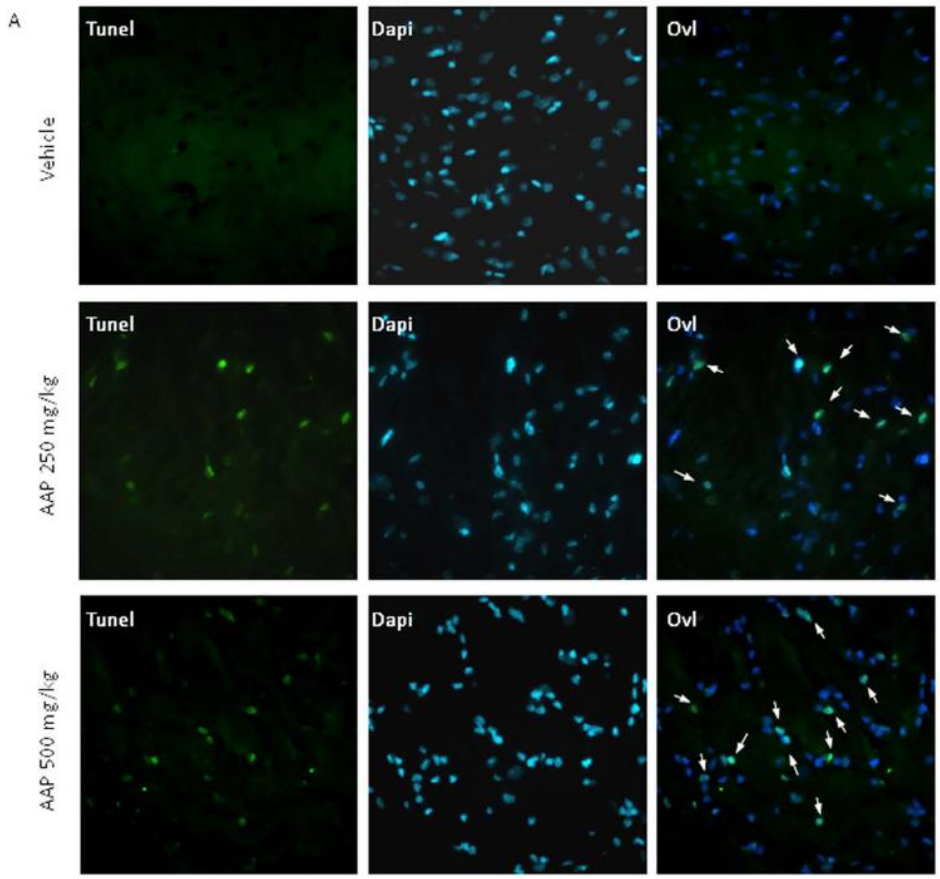


Figure 30. Apoptosis (cell death) in rat brain cortex tissue slices. Tunel is apoptotic cells labeled in green. Dapi is a general nuclei dye that labels all cell nuclei. Ovl is the overlayed images. Reproduced from Posadas et al., 2010.

As discussed, oxidative stress caused by APAP can impact a fetus in a number of ways, including by impacting the fetal brain as visualized in the diagram below. (A) Unconjugated APAP can reach fetal tissues including the brain and is locally biotransformed into the toxic reactive NAPQI by CYP2E1 which is expressed in fetal brain (Brzezinski et al., 1999; Kammala et al., 2022; Posadas et al., 2010). NAPQI would then form covalent protein adducts in mitochondria causing oxidative damage and mitochondrial impairment (B). Maternal metabolism of APAP to NAPQI by CYP2E1 leads to NAPQI formation which travels to the fetal brain and causes damage. However, given the reactive nature of the molecule, this seems unlikely as it would likely rapidly form NAPQI protein adducts (e.g., APAP-cysteine) or adduct to other macromolecules. (C) Maternal formation of toxic NAPQI (at therapeutic or supratherapeutic APAP doses) could cause mitochondrial damage, oxidative stress, inflammation or other pathway activation(s) that transfer signals such as pro-inflammatory cytokines from organs such as liver or placenta (see Koehn et al., 2020). (D) Finally, fetal brain damage could result from maternal and/or fetal APAP detoxification that depletes GSH supplies, leaving the detoxification and antioxidant abilities of the fetus insufficient. Accordingly, otherwise tolerable internal and external exposures become toxic leading to molecular, cellular, and tissue level damage in the developing brain. In addition, NAPQI may be formed in the placenta, and reach the fetal brain (not pictured).

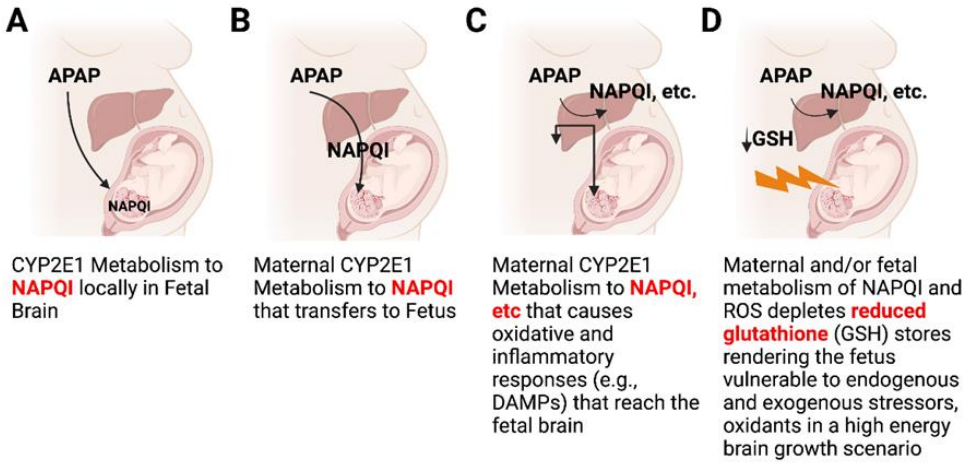

A
APAP
NAPQI
CYP2E1 Metabolism to NAPQI locally in Fetal Brain
B
APAP
NAPQI
Maternal CYP2E1 Metabolism to NAPQI that transfers to Fetus
C
APAP
NAPQI, etc.
Maternal CYP2E1 Metabolism to NAPQI, etc that causes oxidative and inflammatory responses (e.g., DAMPs) that reach the fetal brain
D
APAP
NAPQI, etc.
GSH
Maternal and/or fetal metabolism of NAPQI and ROS depletes reduced glutathione (GSH) stores rendering the fetus vulnerable to endogenous and exogenous stressors, oxidants in a high energy brain growth scenario

Figure 31. Multiple pathways for direct and indirect actions on APAP on fetal brain toxicity. Others likely exist
Created with BioRender.

4. Epigenetic Changes

In addition to its other effects, APAP has been shown to alter epigenetics in exposed offspring (reviewed in Dr. Baccarelli's report). Epigenetics are heritable changes in gene expression and cellular function that do not involve alterations in the DNA sequence. Epigenetic effects encompass modifications to the structure or packaging of DNA, such as DNA methylation and histone modifications, which can influence gene activity and impact cellular responses to toxic exposures.

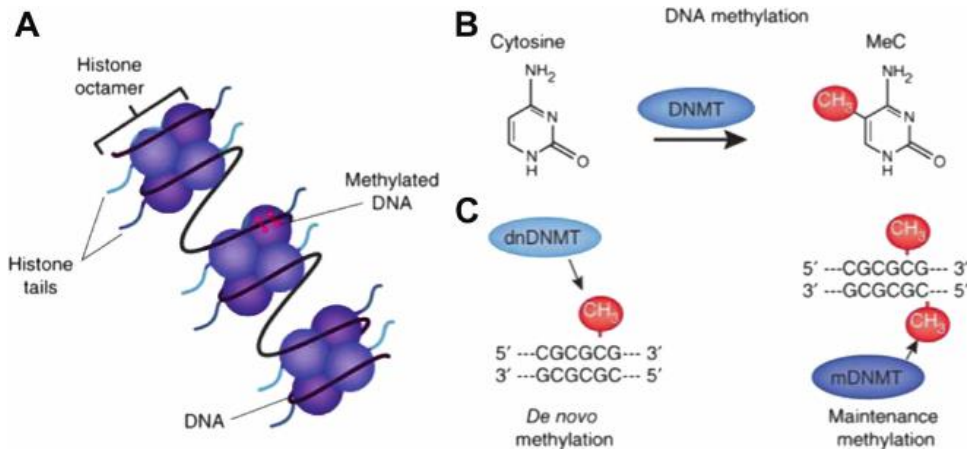


Figure 32. DNA packaging around histone octamers and the process of DNA methylation. DNMT – DNA methyltransferase – the enzyme that adds the methyl group (CH_3) to the cytosine base of DNA.
Reproduced from Chiu et al., 2014.

Epigenetic changes induced by toxicants can effectively silence or activate genes. In addition to altering gene expression patterns, epigenetic disruptions can also perturb normal cellular processes, and contribute to the development of toxicological outcomes, including diseases

and adverse effects on health. Cytokines, growth factors, alterations in hormonal levels as well as release of stress-response and neurotropic factors are some examples of molecules that are modulated by the environment, and which come under the category of epigenome modifiers. Monitoring epigenetic changes allows us to examine another way that the pattern of gene regulation can be perturbed by toxins and can underly complex health risks. Epigenetics are also typically stable enough to indicate a toxic exposure and can potentially cross generations. Modifications to the epigenome can manifest themselves in a number of ways, including, encoding memory of environmental conditions, silencing damaging mutations, or protecting DNA from damage in aging. As discussed above, epigenetic changes caused by toxic compounds can be measured through a variety of tests, including analysis of RNA, histones, and DNA methylation.

Changes in DNA methylation provide an important metric for measuring epigenetic changes caused by toxic exposures. DNA methylation is a vital epigenetic process that involves the addition of a methyl group (CH3) to the DNA molecule. DNA methylation occurs when a methyl group is attached to the carbon atom of a cytosine nucleotide, typically at sites where cytosine is followed by guanine (CpG sites). This modification is catalyzed by enzymes called DNA methyltransferases (DNMTs). The methylation of CpG island in proximity to gene promoters can change gene expression by impeding the binding of transcription factors.

C. Dysregulation of the Endocannabinoid System

In addition to its effects on the body's other molecular and metabolic processes, APAP also affects the endocannabinoid system. The endocannabinoid system (ECS) is a complex signaling system that plays a vital role in regulating diverse physiological and cognitive processes, including neurodevelopment. The ECS also assists in regulating the immune system through CB2 receptors on immune system cells. The ECS consists of endocannabinoids, receptors, and enzymes. Its two primary receptors are CB1 and CB2. CB2 receptors are primarily found in immune cells and peripheral tissues, but CB1 receptors, primarily activated by the cannabinoid anandamide (AEA), are found in the central nervous system. Enzymes, such as fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), are responsible for the synthesis and degradation of endocannabinoids.

The ECS begins to take form early in development, with CB1 and CB2 receptor mRNA detectable as early as pre-implantation in mice (Paria & Dey, 2000). CB1 receptors have been detected at week 14 of gestation in the human embryo, and by week 20 of gestation, selective expression of CB1 receptors is present in the brain (Biegon & Kerman, 2001, Wang et al., 2003). Cannabinoids can fall into several categories, including phytocannabinoids (like the THC found in cannabis), synthetic cannabinoids (produced in a lab), or endocannabinoids. Endocannabinoids, which are produced and occur naturally within the body, are lipid signaling molecules. The endocannabinoid that acts primarily on CB1 is anandamide, an endogenous partial agonist. Anandamide has been detected in the early embryonic stage of human fetal development (Paris and Dey, 2000).

Endocannabinoids modulate the release of neurotransmitters involved in neurodevelopment, such as glutamate and gamma-aminobutyric acid (GABA). The ECS also regulates neuroinflammation and neuroprotection (Battista et al., 2012). Studies have indicated that dysregulation of the ECS during neurodevelopment can lead to developmental abnormalities,

including cognitive deficits and behavioral disorders (Brigida et al, 2017). Animal models have also shown that CB1 is involved in a myriad of behavioral and cognitive processes in neurodevelopment, including memory formation and fearful/avoidant behavior (Marsicano et al., 2002). The ECS is part of the complex web of molecular processes that regulate neurodevelopment, and aspects of it, including FAAH activity, have been linked to tryptophan and serotonin activity.

Normal endocannabinoid function is essential for neurodevelopment. CB1 receptors, in particular, are highly expressed in the developing brain and play a crucial role in regulating neurodevelopmental processes such as neuronal proliferation, migration, and differentiation (Oka et al., 2004). The ECS also regulates neuroinflammation and neuroprotection (Battista et al., 2012).

APAP's action on the endocannabinoid system contributes to APAP's disruption of neurodevelopment. While APAP's primary metabolic route is in the liver, another key APAP metabolite, p-aminophenol (PAP), also plays a role in the body's metabolism of APAP. In the brain, PAP conjugates with arachidonic acid to form a bioactive fatty acid, N-arachidonoyl phenolamine (AM404). In order to convert to AM404, FAAH must be present. Through its actions on TRPV1, a molecule that binds to cannabinoid CB1 receptors and an inhibitor of cellular anandamide uptake, AM404 leads to increased levels of anandamide. Studies have shown that p-aminophenol may be the metabolite most relevant and toxic to developing cortical neurons. For example, in Schultz et al., 2012, the authors found that concentrations of p-aminophenol from 1 to 100 µg / ml produced significant ($p < 0.05$) loss of mouse cortical neuron viability at 24 hr compared to the controls (Schultz et al., 2012). Animal research has also shown that APAP can specifically interact with CB1R agonist WIN 55 212-2 and that disruption in key endocannabinoid enzymes including FAAH (Philippot et al., 2018).

Endocannabinoid dysfunction has been specifically linked to ASD (see e.g. Schultz & Siniscalco, 2019, Siniscalco et al., 2013,). Unbalanced excitatory and inhibitory neurotransmission caused by ECS disruption (Zamberletti et al., 2017). In addition, ECS disruption can result in a lack of control over axon growth, impacting the corpus callosum, the pathway for axons between hemispheres of the brain. Research has demonstrated that axon numbers, diameter, and axon area in the corpus callosum are reduced in the brains of subjects with ASD (Weigel et al. 2018).

Endocannabinoid dysfunction has also been suggested as a causal pathway in ADHD. Endocannabinoid metabolism has been shown to be disrupted in childhood ADHD (Centonze et al., 2009). Genome sequencing studies have also shown genetic variations present in association with ADHD, including variations involved in FAAH metabolism. Changes in CB1 receptors have also been associated with ADHD (Lu et al., 2008). ADHD patients have also been shown to have elevated levels of plasma anandamide (AEA) and sphingosine-1-phosphate (S1Pd18:1) (Brunkhorst-Kanaan et al., 2021. Animal models of ADHD also suggest ECS involvement (Castelli et al., 2011).

Like oxidative stress, endocannabinoid disruption has a close relationship with inflammation. Research has shown a link between pro-inflammatory cytokines in the brain and ASD diagnosis. A pro-inflammatory state in the brain has also been linked to ADHD (Saccaro et al., 2021). Because the ECS provides regulation of immune system cells through CB2 receptors, dysregulation of that ability could lead to increased inflammation in the brain.

It is well accepted that endocannabinoid disruption during pregnancy should be avoided (ACOG, 2018). As previously mentioned, THC use during pregnancy is highly discouraged because it may interfere with normal ECS functioning. Researchers, including Philippot et al., have noted that both THC and APAP cause changes in animal behavior when administered at postnatal day 10 (Philippot et al., 2019; Philippot et al., 2017; Viberg et al., 2014).

D. Effects on Brain-Derived Neurotrophic Factor (BDNF)

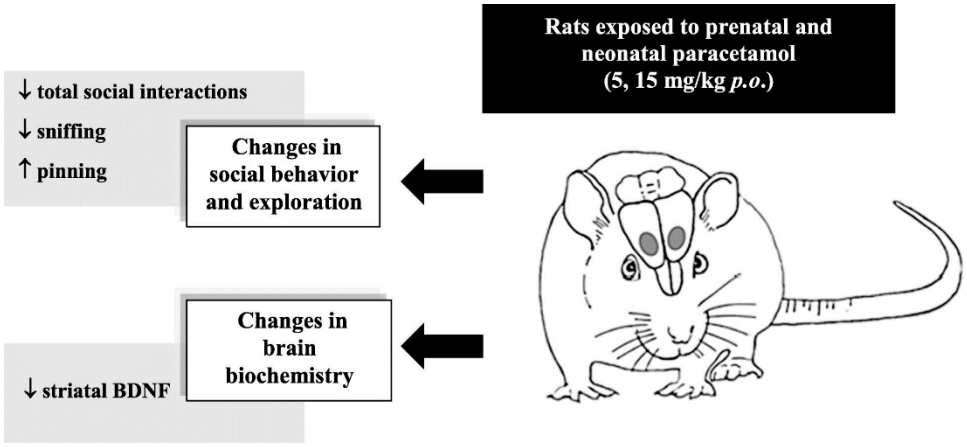

Rats exposed to prenatal and neonatal paracetamol (5, 15 mg/kg p.o.)
↓ total social interactions
↓ sniffing
↑ pinning
Changes in social behavior and exploration
Changes in brain biochemistry
↓ striatal BDNF

Figure 33. Behavioral and chemical effects of in utero APAP exposure related to BDNF in rats. Reproduced from Blecharz-Klin et al., 2018.

Brain-Derived Neurotrophic Factor (BDNF) is a powerful neurotrophin critical to normal neurodevelopment, and particularly important to learning and memory (Miranda et al., 2019). Neurotrophins are proteins secreted by brain cells which use signaling pathways to regulate neurodevelopmental processes. BDNF in particular impacts a wide range of processes in neurodevelopment including neuronal growth and migration (Bathina and Das, 2015). BDNF levels are also key to neuronal survival within the nervous system. In addition, BDNF acts a neurotransmitter modulator. By the second trimester of in-utero development, BDNF is widely presene in the fetal brain and helps regulate the size and speed of brain development in utero (Antonakopoulos et al., 2018).

Evidence in this case, discussed in detail below, demonstrates that BDNF in the developing brain is altered by APAP exposure (Blecharz-Klin et al., 2018, Lalert et al., 2023, Viberg et al., 2014). In studies on BDNF and APAP in animals, animals with altered BDNF also, unsurprisingly, showed behavioral changes. Because of BDNF's central role in neurodevelopment, disruption in BDNF has unsurprisingly been linked to neurodevelopmental disorders in humans, including ASD and ADHD. Specifically, BDNF dysregulation has been linked to ASD etiology in humans (Ohja et al., 2017). Decreased levels of BDNF have also been linked to ADHD in humans (Lie et al., 2015, Corominas-Roso et al., 2013).

F. EFFECTS ON SEROTONERGIC SIGNALING

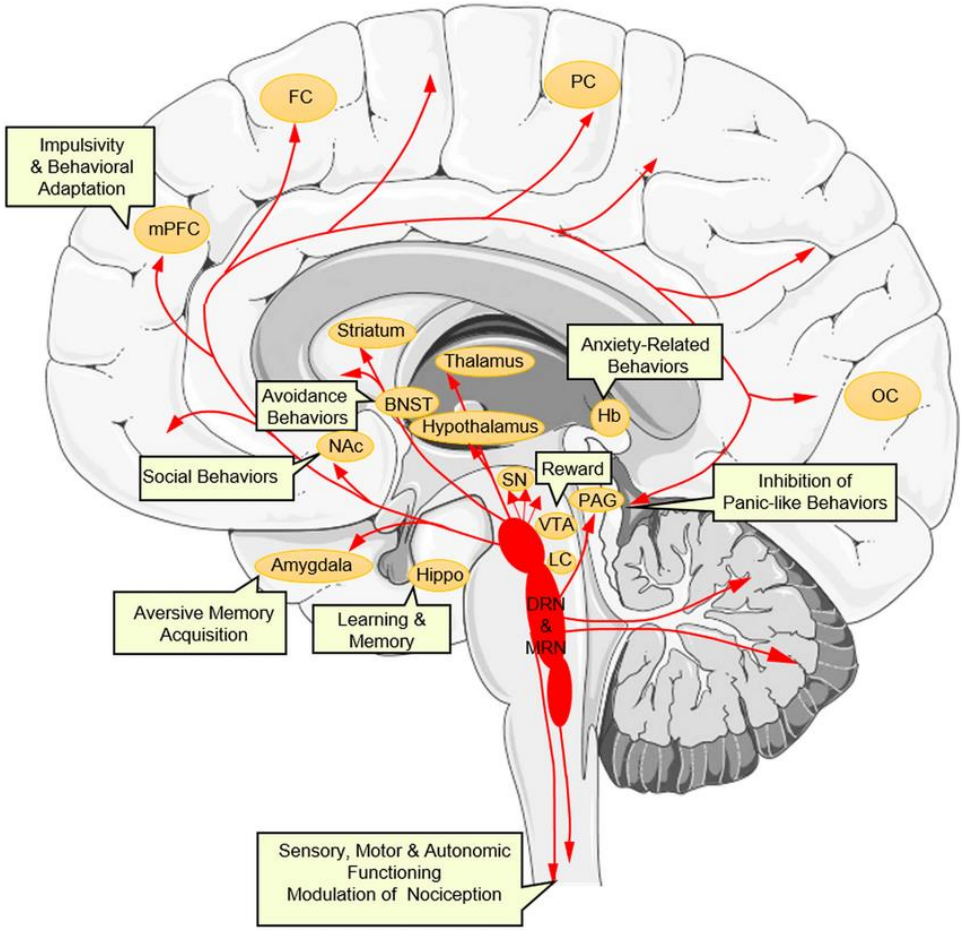


Figure 34. Serotonergic system and its wide-ranging effects. Reproduced from Pourhamzeh et al., 2022.

There is also evidence that APAP may affect normal serotonergic signaling and function in the brain during neurodevelopment. Like the processes described above, normal functioning of the body's serotonergic signaling system are crucial for normal neurodevelopment. Serotonin, also known as 5-hydroxytryptamine or 5-HT, is a neurotransmitter that regulates communication between neurons and influences critical processes in neurodevelopment including neuronal growth, migration, differentiation, synapse formation, and neuroplasticity. Serotonin emerges early in neurodevelopment, with 5-HT neurons beginning to appear when the embryo is 5 weeks old and continue to proliferate during pregnancy.

Serotonin signaling systems are also closely related to other mechanisms discussed in this report. Studies suggest that once APAP is metabolized to form cerebral FAAH-dependent AM404, this metabolite could reinforce the activity of the endocannabinoid system through CB1 receptors, which would in turn reinforce the activity of the bulbospinal serotonergic inhibitory pathways (see e.g., Mallet et al., 2008). Serotonergic activity may also be impacted by BDNF (Siuciak et al., 1996).

Animal studies show that APAP has an effect on serotonin function and signaling in the prefrontal cortex (see e.g., Blecharz-Klin et al., 2017, discussed below). It is well-established that normal serotonergic signaling is critical for neurodevelopment. With regard to ASD, numerous studies have highlighted alterations in serotonin levels receptors in autistic people. ASD phenotypes are associated with disruptions in neuronal migration, synaptogenesis, and circuit formation, all of which are related to serotonin during neurodevelopment. In terms of ADHD, serotonin modulates several key symptom areas of ADHD, including attention, executive functioning, and impulse control. Altered serotonergic signaling has been linked to both ASD and ADHD in humans (Banerjee and Nandagopal, 2015, Kessi et al., 2022, Marotta et al., 2020).

E. Effects on Prostaglandins

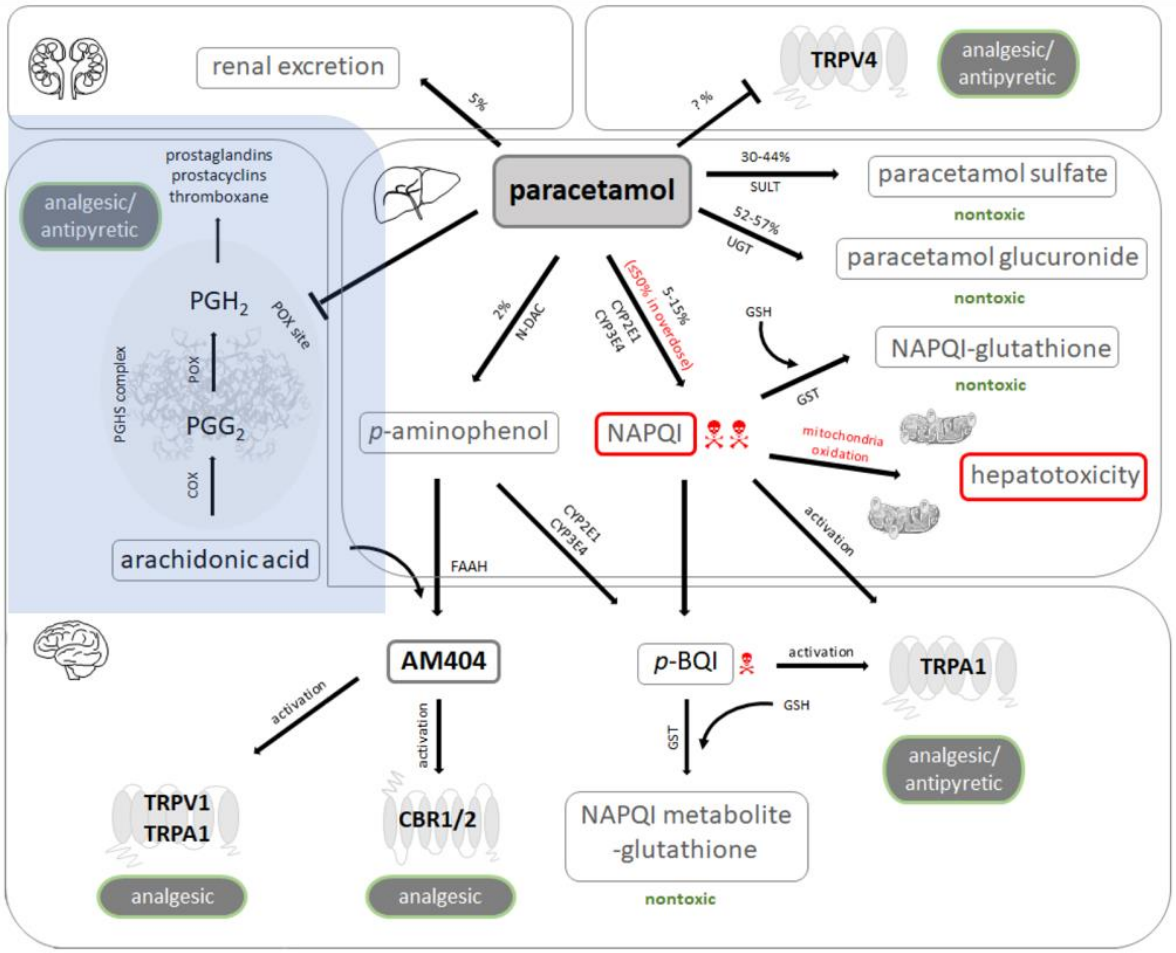


Figure 35. Paracetamol metabolism and pharmacology, with prostaglandin effects highlighted blue. Modified from Buhrer et al., 2021.

APAP is a prostaglandin inhibitor. Prostaglandins are a group of lipid molecules with diverse functions throughout the body, and are derived from arachidonic acid. They can serve as local hormones or signaling molecules (Ali et al., 2006). Because they serve diverse roles and have quite potent effects on cellular function, prostaglandins are targeted by several drugs, including APAP. COX-1 and COX-2 enzymes are responsible for the conversion of arachidonic acid, a fatty

acid found in cell membranes, into prostaglandins. Prostaglandins are present from the earliest stages of human embryonic development and serve neurodevelopmental and neuromodulatory roles that are independent of inflammatory and immune signaling.

It is well known that APAP acts on prostaglandins, and that this action may be responsible for APAP's antipyretic effects. In the context of inflammation, prostaglandins signal peripheral inflammation to the brain such as in states of infection and sickness (Kobrzycka et al., 2019). As with previously discussed mechanisms of action, APAP's effects on prostaglandins are likely interconnected with other processes also affected by APAP. For example, as discussed previously, one of APAP's metabolites is AM404. AM404 is a low-affinity ligand of CB1 receptors, meaning that it binds to CB1 receptors (but with a chemical bond that is not particularly tight). Saliba et al. demonstrated that AM404 prevented proper prostaglandin synthesis in activated microglia by inhibiting COX activity, essentially interfering with the enzymatic conversion of arachidonic acid into prostaglandins.

Prostaglandin receptors are present in a variety of locations in the brain, including the preoptic area and the cerebellum. The cerebellum develops relatively late in pregnancy but is linked to a wide number of outcome and behaviors and processes, including motor control (its best-known function), executive control, social interaction, the integration of sensory information, and memory (Bellebaum & Daum, 2017, Berntston and Schumacher, 1980, Tavano et al., 2007). Nearly all of these processes are directly implicated in ASD and ADHD symptomology. Dean et al., 2012, discussed in further detail later in this report, shows that prostaglandin inhibitors including APAP disrupt neurodevelopment. The authors in that study found that APAP and other prostaglandin inhibitors could act on developmental dynamics in the cerebellum, disrupting normal neurodevelopment.

XI. WEIGHT OF EVIDENCE METHODOLOGY

As introduced in Section III, the WoE approach I used is a systematic review of available data using the following series of steps:

1. Problem formulation (develop hypothesis);
2. Evidence collection (establish lines of evidence and knowledge gaps);
3. Evidence evaluation (determine data reliability, uncertainty and relevance);
4. Evidence weighing (assign weight of evidence); and
5. Evidence integration and reporting WoE conclusions (examine evidence coherence and impact of uncertainty)

(OECD, 2019).

For purposes of this report, I employed my experience performing preclinical studies and peer-reviewing such studies for various publications in my review here and applied the same rigorousness as I do in my capacity as a peer reviewer. Below, I describe each step of my analysis in detail.

A. PROBLEM FORMULATION

The first step in the WoE evaluation, problem formulation, involves creating a hypothesis-based question and defining the scope, goals, and urgency of the assessment, and the acceptable level of uncertainty (OECD, 2019). As described in the Mandate, this report addresses the following hypothesis-based questions: (1) Is the proposal of a causal association between in utero exposure to APAP and neurodevelopmental disorders, including ASD and ADHD, consistent with existing biological knowledge and preclinical literature?; and (2) Is there sufficient preclinical evidence to conclude that that in utero exposure to APAP can cause neurodevelopmental disorders, including ASD and/or ADHD?

Given the prevalence of APAP use in the pregnant population and the substantial incidence of ASD and ADHD, I believe this review is urgent. In risk assessment, as in legal settings, the applicable standard of proof and acceptable level of uncertainty are context-dependent (OECD, 2019). Given that in utero exposure to developmentally neurotoxic substances can, and often does, have significant and enduring consequences, the appropriate standard for this assessment is "'a preponderance of the evidence' or 'greater than 50% confidence'"—i.e., to a reasonable degree of scientific certainty, more likely than not (OECD, 2019).

B. EVIDENCE COLLECTION

The next step in this WoE analysis is the collection of evidence, which includes identifying systematically categorizing evidence and identifying knowledge gaps (OECD, 2019). Evidence collection generally involves devising and documenting a collection strategy, performing a search, and reviewing harvested data for inclusion/exclusion based on scientifically relevant, clearly-identified, and consistently-applied criteria (OECD, 2019). Data is sorted into categories, or "lines of evidence" (LoE) that address the hypothesis (Martin et al., 2018; OECD, 2019). "Assembling the lines of evidence is a key transparency element of WoE that allows stakeholders to ensure all relevant lines of evidence have been considered and helps to identify relevant information gaps" (OECD, 2019). Lines of evidence may include, for example, in situ (field or epidemiological population-based), in vivo (living organism), in vitro (e.g., cell and tissue based), in chemico (chemical reactivity) and in silico (computer modeling) (OECD, 2019).

To collect any potentially relevant literature for my review in this case, I performed a formal PubMed Boolean search using the following terms and operators on May 4, 2023:

("acetaminophen"[Title/Abstract] OR "paracetamol"[Title/Abstract] OR "APAP"[Title/Abstract] OR "Tylenol"[Title/Abstract] OR "4-Acetamidophenol"[Title/Abstract]) AND ("brain"[Title/Abstract] OR "neuro*"[Title/Abstract] OR "behavior"[Title/Abstract] OR "behaviour"[Title/Abstract] OR "neurodevelop*"[Title/Abstract] OR "neurochem*"[Title/Abstract] OR "glia"[Title/Abstract] OR "cognit*"[Title/Abstract] OR "autism"[Title/Abstract] OR "ASD"[Title/Abstract] OR "ADHD"[Title/Abstract] OR "CNS"[Title/Abstract] OR "neurotransmi*"[Title/Abstract]) AND ("mouse"[Title/Abstract] OR "rat"[Title/Abstract] OR "rodent"[Title/Abstract] OR "cell culture"[Title/Abstract] OR "primary culture"[Title/Abstract] OR "organoid"[Title/Abstract] OR "ipsc"[Title/Abstract] OR "ipsc-derived"[Title/Abstract] OR "in vitro"[Title/Abstract]) AND ("gestation"[Title/Abstract] OR

"prenatal"[Title/Abstract] OR "lactat*"[Title/Abstract] OR "in utero"[Title/Abstract] OR "devel*"[Title/Abstract])

I designed a Boolean search string to include the most pertinent keywords within these subheadings, in tabular form, and then in search string form.

DRUG	TARGET	MODEL	PERIOD
acetaminophen	brain	mouse	gestation
paracetamol	neuro*	rat	prenatal
APAP	behavior	rodent	lactat*
Tylenol	behaviour	cell culture	in utero
4-Acetamidophenol	neurodevelop*	primary culture	devel*
	neurochem*	organoid	
	glia	ipsc	
	cognit*	ipsc-derived	
	autism	in vitro	
	ASD		
	ADHD		
	CNS		
	neurotransm*		

This search yielded 122 studies (Appendix B). Based on the relevance metrics described below, eighteen (18) studies were directly relevant to the question of APAP neurological and/or neurodevelopmental toxicity and 8 studies were more broadly or indirectly relevant to APAP neurological and/or neurodevelopmental toxicity. Full abstracts were read for all results. Full articles were read for all articles deemed relevant and for which full text was available. All but one relevant article was available for review as full text. This search term strategy was successful but did not capture all relevant articles that I know to exist. I located other relevant studies that were included from cited studies from other research papers, research that I have cited in my own work, and studies that I found through other search engines. I included relevant articles from across all of these searches in my weight of evidence assessment.

Any studies that did not populate from my search but that I came across in reviewing other documents or otherwise in preparation of my report and that I deemed relevant to the question here were included in my weighing. I also utilized two open-source computational toxicology and toxicogenomics databases, The EPA CompTox Dashboard and The Comparative Toxicogenomic Database, which are described more fully below. This data has been sorted into the following lines of evidence: in vivo, in vitro/ex utero, and in silico.

In addition to peer-reviewed and published studies, relevant evidence may come from other sources (e.g., decisions and analysis reports from regulatory authorities, expert opinion reports, company and/or third party generated studies of a proprietary nature, incident reports, and adverse reactions submitted to regulatory authorities) (OECD, 2019).

I reviewed documents from the United States Food & Drug Administration (FDA) and international regulatory agencies. I reviewed deposition testimony from current and former employees of JJCI. My review also included documents produced by APAP manufacturers, including preclinical studies, mechanistic studies, epidemiological studies, internal risk/causality assessments, and other relevant information.

As a research toxicologist, I routinely rely on data and observations from scientists in related fields—including pharmacodynamics, teratology, and epidemiology—to develop and design studies, interpret results, and reach conclusions about the developmental neurotoxicity of substances. Accordingly, I have reviewed and relied on the general causation expert reports submitted by Andrea Baccarelli, MD, PhD, MPH (epidemiology), Robert Cabrera, PhD (teratology), and Stan Louie, PharmD (pharmacology) in this litigation.

C. EVIDENCE EVALUATION

Next, and perhaps most important, the available evidence is evaluated to determine its relevance and reliability (OECD, 2019). This evaluation is arguably the most important step in the WoE analysis "because it provides the outcomes to determine the weight given to evidence" (OECD, 2019). Accordingly, this subsection provides a detailed description of relevance and reliability considerations.

1. Data Relevance

Data relevance is determined by the degree of correspondence between the available data and the hypothesis-based question under consideration (OECD, 2019). "The determination of relevant versus non-relevant data will be context specific and care should be taken when determining non-relevant data" (OECD, 2019). Because the focus of this report is the developmental neurotoxicity of APAP, studies from other disciplines and studies investigating other, unrelated parameters were screened out at the evidence collection stage, as described more fully above. Additionally, I excluded studies that evaluate only the parental or sexual behavior or F0 or F1 (or future generations) of APAP exposed rodents, as these are not characteristic domains of neurodevelopmental disorders, per se (e.g., Aleixo et al., 2020). During my review of the remaining studies in this stage of the analysis, I excluded additional studies, as they involved either administration of APAP at levels well above therapeutic doses (lethal hepatotoxic dose), administration of APAP to adult rodents at ages that do not correspond to human gestation, and/or were not designed to test DNT.[7] This report provides a comprehensive review of *all* preclinical

[7] **Ishida et al., 2007 *Journal of Psychopharmacology***. The study by Ishida et al. was designed to examine whether APAP influences mouse performance in memory tasks and, if so, determine whether COX-3 inhibition is involved in the mechanism. The study showed that APAP administration did impact mouse performance and spatial memory in the water maze. In the hidden platform (HP) task, mice that received the lowest dose of APAP (15.1 mg/kg) had reduced escape latencies compared to the control group, mice that received the middle dose (69.5 mg/kg) performed substantially the same as the control group, and mice that received the highest dose (302.3 mg/kg) had increased latencies compared to the control group. In the visible platform (VP) task, mice that received the lowest dose of APAP (15.1 mg/kg) had reduced latencies compared to the control group, but there was no statistically significant impact on latencies for mice that received APAP in higher doses or other medications. The authors posited

that the high dose APAP group's impaired performance on the HP task but not on the VP task "may reflect underlying inhibition of endogenous COX-2, and that endogenous COX-2 may modulate spatial memory, which involves hippocampal function." They suggest that the low dose APAP group's enhanced performance in the HP and VP tasks "is attributable to activity of serotoninergic neurons that modulate emotion (motivational and/or attentional processes) and/or motor activity (motor processes) in the striatum and/or amygdala, rather than to the activation of glutamatergic neurons that modulates spatial memory via the hippocampus." This study was in 5-week-old mice which is the equivalence of late adolescent or early adulthood in humans. Accordingly, this is outside of the scope of my report.

Leroux et al., 2010 *Neuroscience*. In the Leroux et al. study, neonatal mice were treated with various NSAIDs (including APAP) for five days before or after intracerebral injection of ibotenate, which produces excitotoxic lesions that mimic the white matter lesions in preterm infants attributable to their preterm status and cortex and basal ganglia damage experienced by full-term infants due to infection or hypoxia. All pups were euthanized on postnatal day 10 and their brains were prepared for analysis. Researchers found that low dose (10 μg) and high dose (100 μg) APAP treatment administered before or after the ibotenate injection did not significantly protect the cortex. Low dose APAP treatment following ibotenate injection "significantly reduced white matter lesion size" compared to controls; however, APAP pre-treatment and high dose APAP post-treatment did not show significant differences from controls. Additionally, pre-ibotenate treatment with high-dose APAP significantly decreased survival, which is "consistent with reports of paracetamol toxicity in human neonates." I deemed this study irrelevant in my lit search because it is evaluating protection from excitotoxic lesions alongside other medications. It does not test developmental toxicity of APAP and does not have an APAP only condition.

Posadas et al., 2010 *PLoS ONE*. (In vitro and in vivo testing was performed in this study, see independent entries for this study). In their in vivo experiments, Posadas and colleagues administered 250 or 500 mg/kg APAP via a single intraperitoneal injection and then measured the plasma and cerebrospinal fluid (CSF) levels of APAP. They wanted to determine if those levels were comparable to the levels that they administered to the cultured cortical cell experiments described in the in vitro experiments discussed in the rest of the paper (and reviewed below in the In Vitro/Ex Utero section of this report). They found that the 250 and 500 mg/kg i.p. injection led to 1 and 2 mM peak (Cmax) plasma levels, respectively, and 1 and 3 mM Cmax in the CSF, respectively which are strikingly high levels. The injections also led to cell toxicity measured by LDH release and apoptosis as measured by TUNEL labeling in brain tissue sections. A limitation of this study is that the route of administration of APAP is not as clinically relevant as other routes, but this does not invalidate the study. Other issues are that the in vivo dosing was not in utero or neonatal rats. They were adult female Sprague-Dawley rats weighing 240–260 g which are solidly adults, placing some limitations on the relevance of the outcome to the neurodevelopmental toxicology as opposed to general neurological toxicology of APAP. Because of the limited relevance to developmental neurotoxicity, I did not include this study in the "weight of the evidence" analysis, although it does show neurotoxicity.

Onaolapo, et al. 2017 *Biomedicine & Pharmacotherapy*. This study compared the relative protective abilities of silymarin, an antioxidant/tissue regenerating flavonoid with hepatic and neuroprotective capabilities, and L-methionine, an essential amino acid shown to possess antioxidant activity, pre-treatment in APAP overdose injuries of the liver, kidney, and cerebral cortex. The authors divided rats into six groups of ten. Five of the groups were pre-treated with vehicle (oral distilled water) at 10 ml/kg, a standard drug (Silymarin at 25 mg/kg) or one of three doses of L-methionine (2.5, 5 and 10 mg/kg body weight) respectively for 14 days; and then administered intraperitoneal (i.p.) acetaminophen at 800 mg/kg/day for 3 days (days 15–17). Rats in the sixth group (normal control) received distilled water orally for 14 days and then i.p. for 3 days. The authors found silymarin or L--Methionine counteracted behavioral changes, reversed biochemical indices of liver/kidney injury, and improved antioxidant activity and conferred some degree tissue protection. Though the authors found these chemicals can protect the liver, kidney, and cortical neurons from APAP-induced injuries, "each offers variable protection to different tissues." I did not include this study in the weighing because it is not designed to test DNT.

Motawi et al., 2019 *Biomarkers*. Motawi, et al. published "Protective effects of betanin against paracetamol and diclofenac induced neurotoxicity and endocrine disruption in rats" in 2019 in the journal Biomarkers. The authors

were evaluating the neurotoxicity of two analgesics, APAP (at a high dose) and diclofenac, and tested the use of betanin to mitigate those effects. I'll only discuss the APAP and control data hereafter. The outcome variables were biochemical and histopathological changes induced by APAP, including oxidative stress and neurotoxicity. Adult male Wistar rats were divided into groups with eight rats in each. APAP (or control) was given by gavage at 400 mg/kg for 28 consecutive days. There was a notable reduction in brain serotonin, noradrenaline, and dopamine levels in those exposed to APAP compared to the controls. Additionally, MDA, a marker of oxidative stress, was significantly increased in the APAP treated rats compared with the controls. APAP also had an effect of decreasing serum NrF2 and brain GSH content, HOX-1, POX-1, SOD, and CAT activities compared with controls. DNA fragmentation measured using the Comet assay was increased in the APAP exposure condition. Finally, brain Sections of rats exposed to APAP showed severe edema and hyalinized axon with large numbers of inflammatory cells between neurons compared with normal histological structure of control rats and ameliorated effects (slight axonal edema and less inflammatory cells). Given that this study used adult rats and did not test APAP in a developmental context, its relevance for neurodevelopmental disorders could be limited. Accordingly, I do not include this study in the weighting.

Vigo et al., 2019 *Biochemical Pharmacology*. Vigo and colleagues administered a single intraperitoneal injection (abdominal) of APAP (1g/kg) (or a vehicle control) to adult male rats with the goal to model hepatic injury related encephalopathy. Rats were either measured in a behavioral test of locomotion or euthanized to evaluate blood, liver, and brain markers. APAP-dosed rats showed the expected histological liver/hepatocellular necrosis with elevated plasma ALT and AST. However, liver functional markers including PTT, aPTT and FV and ammonia levels were all unchanged showing that the dose administered was not sufficient to cause acute liver failure. APAP administration was associated with brain edema (water-associated swelling) as well as dopamine (DA) and DA metabolite level declines in multiple brain regions. APAP treated rats showed lower locomotor activity in an open field test. APAP exposure was also associated with reduced levels of tyrosine hydroxylase (TH) in the dorsal striatum and the nucleus accumbens. Tyrosine hydroxylase is an enzyme involved in the synthesis of dopamine. Dopamine is relevant to movement control and reward-related processes. The reduction in TH levels suggests that APAP treatment may have impacted dopamine synthesis in these brain regions, potentially affecting motor function and reward-related behaviors. As TH is the rate limiting enzyme in DA synthesis the DA and locomotor changes could be attributed to this effect on TH in this study. APAP was also associated with changes in the expression and nuclear translocation of NRF2 – a general indicator of widespread oxidative stress given its role as un upstream transcription factor regulating the expression of antioxidant and detoxification systems in cells. This supports that the APAP exposure caused brain oxidative stress in all regions tested except for the dorsal striatum. This study provides mechanistic insights into how APAP could lead to neurodevelopmental toxicity and alter neurochemistry in a perinatal context but has limited relevance as it is in adult animals and the dose of 1g/kg (1000 mg/kg) is high, and route of administration is not ideal. Accordingly, I did not include this study in my Weight of Evidence analysis.

Viswanathan et al., 2019 *Journal of Ethnopharmacology*. In a study published by Viswanathan and colleagues, 4-5 week mice were administered various combinations of Centella asiatica methanol extract (CAM) and Paracetamol to evaluate whether CAM can protect mice brains from Paracetamol-induced toxicity. The authors administered a single 500 mg/kg oral dose of APAP. They did not say if this was given by gastric gavage or given in the drinking water. Nevertheless, this is an overdose study. One hour later they euthanized the mice and looked at brain homogenates and brain sections for evidence of radical stress and histopathological damage and to see if the natural produce could abrogate that change, which it appeared to have been able to do under certain circumstances. I exclude this study from the weight of the evidence because I do not find it relevant given the exceptionally high dose and the fact that this was performed in 4-5-week old animals which are outside of the developmental scope of interest.

Pinto et al., 2020 *Neuron*. Pinto (2020), as discussed previously in this report, also examined APAP's effects on neurodevelopment. While the study found that APAP was protective in the Down's syndrome model, it showed neuroinflammatory and electrophysiological alterations in wild type control mice when administered at dosage of 100mg/kg in the early pre-weaning period or ranging to early adulthood. While this was not the focus of the authors,

DNT studies with relevant information—i.e., those that have found a positive association between in utero APAP exposure and altered neurodevelopment, and those that have not.

Developmental neurotoxicity risk assessments virtually always include toxicology data from animal studies because the required experiments cannot ethically be performed in humans. Accordingly, DNT data from animal studies is routinely utilized and extrapolated to human populations. This process is well-established and scientifically validated, as discussed in further detail below. Other differences between animal studies and real-world exposure in human populations will require additional extrapolation (e.g., route of administration, translationally relevant dosing, etc.) Many of these differences can be accounted for in the study design and/or analyses, as described below.

2. Data Reliability

The reliability of evidence is determined by assessing the quality and the quantity (or "sufficiency") and consistency of data across the LoE, with due consideration for the uncertainty associated with each of these elements (OECD, 2019). In chemical assessment, "uncertainty" generally refers to "imperfect knowledge concerning the present or future state of an organism, system, or (sub)population under consideration," though it may also refer to a lack of certainty that results from unclear communication, manipulation, or errors in reporting data (World Health Organization-International Programme on Chemical Safety (WHO-IPCS), 2018; OECD, 2019). In some circumstances, uncertainty can be described using a quantitative approach. However, chemical risk assessments generally utilize a qualitative approach to uncertainty analysis "simply because of the lack of adequate data to support statistical models" (OECD, 2019). Regardless of

---

they had appropriate controls that permitted these comparisons and these conclusions. I exclude this study from the weight of the evidence because of the age of the animals tested.

Pereira, et al., 2020 *Reproductive Toxicology*: Pereira, et al. studied whether maternal APAP exposure during pregnancy and during pregnancy and lactation can affect reproductive and behavioral parameters in male offspring. Female Wistar rats were treated daily by gavage with water or 350 mg/kg APAP during gestation or during gestation and lactation periods. Male offspring were euthanized at three separate ages (PND 21, 50, and 120), representing childhood, adolescence, and adulthood respectively. Body weight, anogenital distance, sexual behavior, copulatory behavior, and sexual incentive motivation were evaluated. Blood samples were collected at PNC 21, 50, and 120 from the euthanized rats for plasma testosterone quantification and testes were removed. The authors analyzed biometric parameters and histology of the left testis. Multiple sperm parameters were analyzed. In male offspring exposed to APAP during pregnancy, there was a significant reduction in latency to the first ejaculation and an increase in the number of ejaculations compared to the control group. In the male offspring exposed to APAP during gestation and lactation, there was a significant increase in latency to the first post-ejaculatory intromission compared to the control group. Exposure to APAP during pregnancy also increased the volume and total length of the seminiferous tubules, increasing the absolute testis weight of the male offspring. The authors concluded that the results showed that "maternal treatment with APAP affects sexual behavior and reproductive parameters of male offspring due to impaired hypothalamic sexual differentiation." They found these results especially seen in those rats exposed only during pregnancy. I did not include this study weighing because reproductive and sexual behavior outcomes are outside the scope of my report.

its definition and characterization, uncertainty must always be considered in the appropriate context. "The Precautionary Principle states that in cases of serious or irreversible threats to the health of humans or ecosystems, acknowledged scientific uncertainty should not be used to postpone preventive measures" (The American College of Obstetricians and Gynecologists (ACOG, 2021).

Data sufficiency and consistency are related concepts. "Sufficiency" refers to the quantity of data that addresses the hypothesis/problem and "consistency" refers to the level of consensus and concordance among the data in a particular LoE. (OECD, 2019). "[T]he highest level of data reliability is achieved when, given the context of the question, there is a sufficient amount of quality data that are internally consistent and can be shown to support the hypothesis with a plausible explanation" (OECD, 2019).

3. Data quality

The quality of relevant studies is generally determined by the degree of transparency in documenting the study's methodology, analysis, and results (OECD, 2019). Data quality also depends on the degree to which potential methodological bias (e.g., information and selection bias) is considered. "Two types of assessment methods are presented in the literature, *i.e.*, those with or without quantitative scoring" (Martin et al., 2018). There are numerous approaches to assessing data quality – some include quantitative scoring, others do not (Martin et al., 2018). Whatever approach is selected, it must be applied consistently and transparently to all data under consideration (OECD, 2019). For this report, I have used a coarse quantitative scoring system to enable objective comparisons between the studies reviewed.

Accordingly, for each study included in my review, I carefully reviewed the data quality to determine the level of concern for adverse neurodevelopmental outcomes in humans prenatally exposed to APAP. Further, I utilized a coarse scoring scheme to evaluate key parameters of importance for each relevant publication to use in a weight of evidence analysis. Table 1, below, was used to score each relevant publication. The applicable score is provided with the narrative review of each study. Parameters such as sample size were considered for numbers in terms of whether they were powered sufficiently or not, but also whether the paper clearly stated the sample size and the biological and technical replicates. This scoring scheme gave roughly equal weight (up to two points) to all parameters except for "outcomes" which had up to three points because the outcome variables need to be relevant to measurable and meaningful neurodevelopmental toxicity, impairment, perturbation, or insult. Once all of the pertinent papers were scored, these scores were compared on balance on the spectrum of their score of negative and positive indicators for evidence of developmental neurotoxicity.

Table 1

Parameter	**Scoring (TEMPLATE)**	**Score**
Direction of Effect	[APAP DNT (+) or not DNT (-)]	
Controls	[Insufficient (0) Acceptable (1) Good (2)]	
Sample Size	[Insufficient (0) Acceptable (1) Good (2)]	
Outcomes	[Poor quality (0) Moderate Quality (1) Good Quality (3)]	
Multi-Dose	[One dose (0) Multi dose (1)]	
Blinding	[No blinding mentioned or conducted (0) Some Blinding (1) All "Blindable" Parameters Blinded (2)	
Bias/Conflict Flag	Asterisk, does not alter score	**Total**

In evaluating the quality of the relevant DNT studies and assigning scores according to the parameters listed above, I considered numerous features of the studies, including:

a. Study Design

The quality of a DNT study is determined by analyzing numerous factors, including the use of appropriate experimental procedures and controls. Various scientific and regulatory bodies have promulgated guidelines for DNT studies which include general test strategies and, when appropriate, recommendations for specific criteria.

Animal Model. When evaluating the suitability of the animal model selected for DNT testing, several factors should be considered, including but not limited to the test substance's pharmacokinetic profile, the animal model's relevance to humans and sensitivity to test substance; and the test animal's suitability to the test parameters and endpoints. The rat is the preferred animal model for DNT testing (Tyl et al., 2008), though other animals, particularly mice, are routinely and reliably used (International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH), 2020). Table 2, adapted from ICH S5 R3, identifies the advantages and disadvantages of developmental and reproductive testing in rats and mice.

Table 2

Routine Species

Species	Advantages	Disadvantages
Rat	Well-understood biology	Different placentation to humans (e.g., timing, inverted yolk sac)
	Widely used for pharmacodynamics and drug discovery	Dependence on prolactin as the primary hormone for establishment and maintenance of early pregnancy, which makes them sensitive to some pharmaceuticals (e.g., dopamine agonists)
	Robust reproductive capacity with short gestation	Highly sensitive to pharmaceuticals that disrupt parturition (e.g., nonsteroidal anti-inflammatory drugs in late pregnancy)
	Large group sizes and litter size	Less sensitive than humans to fertility perturbations
	Data available from repeated-dose toxicity study	Limited application for foreign proteins • Limited or no pharmacologic activity • Potential impact of immunogenicity
	Suitable for all stages of testing	
	Widespread laboratory experience and availability	
	Extensive historical data	
Mouse	Similar advantages to rats	Similar limitations to rats
	Genetically modified models available or can be generated	Small fetus size and tissue volumes
	Surrogate molecules are often available	Stress sensitivity
	Uses small amounts of test material	Malformation clusters are known to occur

Dose(s). The substance at issue here, OTC APAP for adults, is generally orally administered and has a maximum daily dose of 4 grams (LiverTox, 2016). Doses higher than 4 grams in a 24-hour period are well-known to induce hepatotoxicity in human adults, although studies have shown that APAP can induce liver toxicity even at clinically relevant doses (Sabaté et al. 2011). Given that research animals are smaller, have different basal metabolic rates, and have different surface area to volume ratios, scaling factors are generally applied to translating human

doses. In rat and mouse studies, anywhere from 5 to 500 mg/kg are administered to study APAP's effects, and doses ranging from 350-3000 mg/kg are used to cause liver injury or liver failure at the higher range.

Numbers and Durations of Exposures. Philippot et al., 2021 states "There are always difficulties translating findings from animal models to humans. In humans, key developmental processes are often prolonged over longer time spans compared to mice, thus weeks of exposure in mice may correspond to extreme exposure situations in a human setting. Therefore, the single-day exposure model used... may, in terms of translational relevancy between mouse and human, correspond to more realistic human exposures compared to rodent models where exposures last for several consecutive days/weeks." The choice to administer the chemical multiple times or once is challenging and depends on whether the researcher has an a priori hypothesis about the critical window of injury. If so, then targeting a shorter time period for exposure will make sense. However, if a drug is thought to have potential harms via multiple pathways and during various key points in neurodevelopment, covering more of the developmental window can be more suitable to establish the effect. Once the effect is reliably established one can design follow up studies to resolve the critical window(s) that might underlie the causal damage. This approach is further warranted since many pregnant people report using APAP for extended periods of time throughout their pregnancies.

Method of Administration. There are numerous routes of exposure/methods of administration used in preclinical DNT studies. Selecting the method of administration is often informed by, e.g., the anticipated route of administration in humans, the purpose of the individual study, the particular animal species used, the target tissue or organ, and the test compound's characteristics. The DNT studies I reviewed for this report utilize the following methods of administration described in Table 3, below.

Table 3. Created with Biorender.

Method of Administration	Description	Image
Intraperitoneal Injection	Intraperitoneal injection is a method of administration in which test substances are injected into the peritoneal cavity, the space within the abdomen. This method allows for relatively rapid absorption and systemic distribution of the compound.	
Subcutaneous Injection	Subcutaneous injection is a method of administration in which substances are injected into the subcutaneous layer of the skin. This method enables slower and sustained absorption, as the compound is gradually released into the bloodstream.	
Oral Administration	In oral administration, substances are provided through the oral route, either through gavage (described in more detail below) or mixed with food or drinking water. Oral administration allows for systemic absorption through the gastrointestinal tract.	
Gavage	Gavage is a form of oral administration in which a gastric tube is used to deliver substances directly into the subject animal's stomach. Animal restraint is required.	

Orally-administered APAP products are the focus of my inquiry and, as such, tests that utilize other routes of administration require an additional degree of extrapolation. However, the pharmacokinetic and toxicokinetic properties of APAP are well understood such that animal studies involving non-oral administration can be reliably incorporated into the DNT assessment of orally-administered APAP in human populations (Tyl et al., 2008).

Number of test subjects. The appropriate number of animals for control and experimental groups should be determined by reference to the known variability of test methods and measurement procedures. "[A]ttempts to establish a single number of animals as the group size for all tests and procedures carried out throughout the study, based strictly on the variability of the test method or procedure, becomes very complicated, if not virtually impractical. In practice, the objective is for a sufficient number of pregnant animals to be exposed to the test substance to ensure that an adequate number of offspring are produced for neurotoxicology evaluation" (Tyl et al., 2008).

Test conditions and sequencing. Equivalent test conditions and procedures should be used with all experimental groups, the duration of behavioral tests should be consistent across experimental groups and sufficient to evaluate the target behavior, and serial behavioral tests should be sequenced in a manner that ensures individual tests do not inappropriately impact others (Tyl et al., 2018).

b. Controls

Positive controls. A positive control—i.e., a known neurotoxic agent that is not the test substance—can provide added confidence that the DNT test was designed and executed in a manner that appropriately and reliably detected relevant outcomes. In a Toxicology Hazard Assessment, such positive control data is essential when validating the test. Conversely, in the WoE approach, the absence of positive control data does not necessarily disqualify a study from consideration unless there is a reason to believe that the experimenter or lab is not capable of reliably measuring the outcome of interest.

A positive control substance and test substance should interfere with the same neurobiological system. "Obviously, the neurobiological target of the test material would have to be known a priori" (Tyl et al., 2008). When the neurobiological target of the test substance is the subject of emerging science and significantly but incompletely understood, such as APAP, identifying a positive control presents a significant challenge that has little upside in the WoE context.

Indeed, positive control data is meant to verify that the test laboratory "can effectively demonstrate effects that are qualitatively and quantitatively consistent with those reported in other laboratories for the same agent, at similar doses, and under comparable conditions. This outcome provides added confidence that the absence of effects due to a treatment accurately reflects the situation rather than being due to inadequate implementation of a valid test method (i.e., a false negative)" (Tyl et al., 2008). An evaluation that considers all relevant data—including data that lacks positive control and is, therefore, more likely to include false negatives—creates a bias toward finding no developmental neurotoxicity. Because this potential bias favors the position of APAP manufacturers and stands in opposition to my hypothesis, I have included data presented in the absence of positive controls.

Vehicle (negative) controls. Then, reviewers analyze data from the concurrent vehicle (negative) control group, which is exposed to a toxic agent with no neurotoxic potential via the same route of administration used in the experimental groups. Statistical analysis (e.g., standard deviation, coefficient of variation) should be used to determine whether the control group showed excessive variability, which indicates deficiencies in study design and/or execution. Next, reviewers compare data from the male and female control animals to look for (1) appropriate ontological profiles, (2) presence of normal behavioral characteristics, and (3) male and female behavior consistent with expectations. Generally speaking, a negative control can help understand the specificity of the effects of one's test article (drug). However, this is often superfluous to many study designs, and in the case of in vivo toxicology testing, can create unjustifiable numbers of animals.

If concurrent vehicle (negative) control data demonstrates suitable study design and execution, data from experimental groups can be reviewed for treatment-related effects.

D. TREATMENT-RELATED EFFECTS

"As with all scientific data interpretation of effects, a balance between statistical and biological significance is required" (Tyl et al., 2008). Statistical differences between control and experimental groups should be examined in detail. But biological significance can, and often does, exist in the absence of statistically significant differences between control and experimental groups. A modest difference from control may be biologically and toxicologically significant—even if it is not statistically significant—when a toxicity response pattern and other evidence of toxicity are present. "Within this context, due consideration must be given to the variety of possible toxicity response patterns. Several important response patterns include: 1) the possibility of gender differences in response (i.e., one sex may be more susceptible or differently susceptible than the other); 2) paradoxical dose response curves (e.g., U-shaped); 3) kinetic effects that may impact actual tissue dose (e.g., absorption plateaus that may explain flat dose-response curves); and 4) treatment-related effects that are specific to a given test parameter" (Tyl et al., 2008).

Evidence of neurotoxic effect can also be subtle and somewhat obscure. For example, if the experimental group has increased variability in a test parameter—even if the mean value is equivalent to the control group—this may be evidence of neurotoxic effect. "Therefore, variability can be interpreted as an endpoint itself rather than an indicator of study quality or reliability, and may be the first or most sensitive indicator of a response" (Tyl et al., 2008). These nuances demonstrate why guidance documents, including Tyl et al., repeatedly explain that "[p]roper interpretation involves expert judgment that takes into account both the biological and statistical significance of the results" (Tyl et al., 2008).

1. Consistency Over Time

The biological relevance of treatment-related DNT effects is informed by the temporal relationship between exposure and outcome. For example, neurodevelopmental changes observed at a single point in time or in an inconsistent pattern may have limited biological significance; however, the same changes may be considered biologically significant "when the pattern is consistent with the ontogeny of the endpoint, especially if the changes are associated with a similar pattern in other endpoints (e.g., if there were also developmental delays in a number of other development endpoints indicating likely overall delays in development)" (Tyl et al., 2018). Importantly, "[l]ack of information about a biologically relevant pattern should be considered as an uncertainty and not a negative in a weight of evidence evaluation. Lack of a known underlying biological mechanism is NOT a reason to discount the finding" (Tyl et al., 2008).

2. Consistency Across Doses.

The shape of the dose-response curve is also relevant to the evaluation of DNT data. When increasing doses yield increasing effects in a linear fashion, that enhances confidence that there is a causal relationship between the exposure and the observed effect. When the dose-response curve is nonlinear or multimodal, the DNT data's biological relevance should be evaluated in the context

of other factors, "including the occurrence of other significant treatment-related effects" (Tyl et al., 2008).

3. Consistency Between Sexes.

When statistical analysis reveals sex-specific differences in a study parameter, determining the biological significance of this finding requires careful consideration. These disparities may be caused by hormonal differences, varying sensitivity of the target organ (e.g., ovaries, testes, brain), differences in how each sex responds to a test environment, or differences in absorption, distribution, metabolism and excretion (ADME). Data that clarifies these disparities may exist but is not required in order to find sex-specific differences in DNT endpoints. Where statistical analysis demonstrates no significant sex-related differences in DNT-related endpoints, males and females can be treated as a single group in the analysis (Tyl et al., 2018).

4. Concordance with Other Indices Within Effects Category

DNT studies often evaluate numerous parameters that involve interrelated functions, organs, and apparatuses. Furthermore, the tiered organization of the CNS means that many DNT parameters rely on the proper functioning of various behavioral systems. In order to meaningfully interpret the treatment-related effect of an exposure, it is important to assess the functional integrity of all involved pathways and thereby isolate the effect in question. For example, if there are changes to brain morphometry (i.e. changes in size, volume, and shape characteristics of different brain structures and tissue types), it should be determined whether consistent histopathologic findings also exist (e.g., cell death, changes in brain weight, etc.). (Tyl et al., 2008) "One indicator of insult, which may provide the concordance with altered behavioral indices, is changes in neurochemicals within the brain" (Tyl et al, 2008).

5. Concordance with Other Effects at Comparable Doses.

Treatment-related changes in DNT endpoints should be evaluated in the context of general or other organ toxicity. The presence of other toxicity supports a finding that exposure adversely affects development, even if it is not necessarily neurotoxic. Conversely, a unique or intermittent finding may diminish the biological relevance of this effect; however, "just because an isolated finding cannot be mechanistically explained, this should not be used, in and of itself, to ignore or discount the effect" (Tyl et al., 2008). "Since many of the assessed parameters evaluate disparate functions, and since developmental effects can be specific to particular developmental stages, lack of consistent effects across time points does not necessarily indicate that the effects are not treatment related" (Tyl et al., 2008).

E. WEIGHING EVIDENCE

For each Line of Evidence, the relevance and reliability of data is considered, and a weight is assigned. A qualitative approach is generally used to describe the weight of a particular LoE, though quantitative paradigms do exist (OECD, 2019 ("[N]umerical scores can be used to weigh lines of evidence, but 'those scores are numerical but not quantitative.' Thus, there is no advantage to a numerical system; in fact, it may appear to be more rigorous than is possible.")). Again, regardless of what approach is selected, transparency and consistency are of paramount importance.

Symbols or numerical values that correspond to qualitative descriptions may be used, but they "are not essential if a fully qualitative approach is preferred" (OECD, 2019). I developed the following paradigm, based largely on the OECD guidance document, to qualitatively score each LoE's reliability and relevance as low, moderate, or high. Those scores are then combined to assign an overall weight to the LoE.

Line of Evidence	Reliability	Relevance	Weight Assigned
LoE	[low, medium, high]	[low, medium, high]	[low; low to moderate; moderate; moderate to high; high]

Studies that support the conclusion that APAP exposure causes neurodevelopmental disorders received a positive total score and those that oppose the conclusion received a negative total score. Some studies were not included in the weighting because they were ultimately not relevant to the inquiry, as will be explained. For the studies that were weighed, no study, negative or positive, received a score of zero. Studies ranged in weight, on both the positive and negative sides, from a score of 3.5 to 9. The differentiator between many studies was the strength of the outcome, the factor with the greatest point range.

F. INTEGRATING EVIDENCE

The last step in the WoE analysis involves integrating the LoEs. There are three main objectives for evidence integration, as described in the OECD guidance document:

- "To check the consistency of different lines of evidence, that is the extent to which the contributions of different lines of evidence drawing a specific conclusion are compatible
- "In case of inconsistencies, to try to understand and explain the reasons for them, possibly deciding if more than one answer to the formulated problem is plausible
- "To reject cases of unacceptable or inconsistent outliers to conclude on the WoE based on consistency and quality."

(OECD, 2019).

To that end, there are two primary considerations to be explored during the evidence integration process: (1) "coherence (or in other words consistency) of the lines of evidence for a property/endpoint," and (2) "the relationship of the collected evidence with the WoE outcome(s)" (OECD, 2019).

Identifying and properly interpreting treatment-related effects is challenging because DNT studies often assess several parameters that are associated with the same function, organ or apparatus, while others are part of a battery. Many tests rely on multiple behavioral systems functioning properly, so accurate interpretation requires evaluation of the animals' appearance, behavior, and motor functioning. Furthermore, the correlation and functional impact of the various clinical, functional, biochemical, and morphological parameters are not always understood. Nevertheless, "in the interest of public health protection, these data may be used for regulatory purposes even in the absence of correlations across endpoints" (Tyl et al., 2008).

It is also important to distinguish toxicity from neurotoxicity and adaptation from dysfunction. In some cases, determining that a change in behavior is adverse dysfunction – not adaptation – is relatively straightforward. But that is not always the case. For example, transient treatment-related effects may be adaptive or non-adverse (i.e., within the normal range of physiological equilibrium); however, "transient" changes during perinatal development can lead to long-term adverse consequences that are not necessarily evaluated in the study being reviewed. Ultimately, for purposes of regulation and public health, "an effect of constellation of effects can be viewed as a continuum from non-adverse to adverse with the designation best determined on a case-by-case basis in the context of the presence (or absence) of other effects" (Tyl et al., 2008).

The biological relevance of behavioral changes that indicate treatment related DNT effects should be evaluated in the context of other neurotoxicity parameters (e.g., changes in neurotransmitter levels, mitochondrial dysfunction) and other organ toxicity in the dam or offspring (e.g., hepatotoxicity) where possible. Identifying changes in other neurotoxicity parameters generally increases confidence that the treatment has induced developmental neurotoxicity. But, importantly, "the presence of other non-neurotoxic effects is not sufficient to discount an apparent neurotoxic effect as being secondary to generalized toxicity" (Tyl et al., 2008). Furthermore, dose-related developmental neurotoxic effects observed in the absence of other neuro- or general toxicity should not be dismissed as biologically irrelevant.

Determining whether maternal toxicity exists in dose(s) impacting offspring is valuable, as treatment-related toxic effects on the dam may be causally related to the effects observed in the offspring (secondary toxicity). But even in the absence of data clarifying this issue, "a conservative interpretation for public health protection would be that any indication of offspring toxicity should be considered an offspring-specific treatment-related effect" (primary toxicity) (Tyl et al., 2008). "This same rational may also be used for interpreting DNT data in the presence of other general treatment-related toxicity in the offspring[,] such as body weight" (Tyl et al., 2008).

Determining whether offspring are experiencing primary or secondary toxicity is helpful for understanding the treatment's mechanism of action and, by extension, the applicability of cross-species extrapolation. "However, the determination of primary versus secondary neurotoxicity has no bearing on adversity (i.e., both situations are adverse)" (Tyl et al., 2008).

In sum, the following analysis is based on an established Weight of Evidence methodology. The preclinical evidence weighed here comes from peer-reviewed studies and is the type of evidence that neurotoxicologists commonly use to assess the toxicological association between an exposure and a neurodevelopmental disorder. In keeping with the ultimate goal of a WoE assessment, the scoring system I employ renders my analysis and conclusions easily testable. Accordingly, this analysis yields an opinion on biological plausibility that is relevant to the causation question raised in this case and that I can reliably offer with a reasonable degree of scientific certainty.

XII. APAP DEVELOPMENTAL NEUROTOXICITY STUDIES

Published preclinical evidence since at least the 1980s has demonstrated that, at relevant doses, APAP causes damage to nervous system tissues when exposed during prenatal or prenatal

equivalent periods. This published preclinical data has addressed APAP's effects in rats, mice, and embryos/cells in culture, which will be addressed in turn and in chronological order below.

A. In Vivo DNT Studies

1. Rat Studies

Year	Author(s)	Strain/ Stock	Sex	Exposure Route	APAP Dose mg/kg/day[#]	Score	In FDA review?
2001	Beck et al.	Sprague-Dawley	Unspec	Oral	0, 125, 250, 500	5	No
2012	Dean et al.	Sprague-Dawley	Both	Subcutaneous injection	0, 40	5	No
2015	Lichtensteiger et al.	Wistar	Both	Gavage	0, 360	6	No
2015a	Blecharz-Klin et al.	Wistar	Male	Oral	0, 5, 15	5.5	No
2015b	Blecharz-Klin et al.	Wistar	Male	Oral	0, 5, 15	5.5	No
2016	Blecharz-Klin et al.	Wistar	Male	Oral	0, 5, 15	5.5	Yes
2017	Blecharz-Klin et al.	Wistar	Male	Oral	0, 5, 15	7.5	No
2018	Blecharz-Klin et al.	Wistar	Male	Oral	0, 5, 15	7	No
2018	Saeedan et al.	Wistar	Unspec	Subcutaneous injection	0, 50	3	No
2019	Blecharz-Klin et al.	Wistar	Male	Oral	0, 5, 15	4.5	No
2020	Klein et al.	Wistar	Both	Gavage	0, 350	7	No
2020	Koehn et al.	Sprague-Dawley	Both	Intraperitoneal injection	0, 7.5, 15, 30	7	No
2021	Rigobello et al.	Wistar	Both	Gavage	0, 35, 350	8	No
2021	Suda et al.	Sprague-Dawley	Both	Subcutaneous injection	0, 14.7 mg/kg/5 hrs.	4	No
2022	Herrington et al.	Sprague-Dawley	Both	Subcutaneous injection	0, 51.97	4.5	No

[#]*Doses are listed in mg/kg/day unless otherwise noted.*

Beck et al., 2001 *Toxicological Sciences*. The purpose of the study was to evaluate how prenatal APAP exposure affected reduced GSH abundance and distribution throughout the embryonic tissue. Beck and colleagues administered a single oral dose of 125, 250, or 500 mg/kg APAP via oral gavage to pregnant rats at gestational day 9, and embryos were collected at 0, 6, 12, 24, or 48 hours later. The exposure conditions were not associated with any overt embryonic malformations. To measure the abundance of free thiols such as GSH, they prepared sections of embryos that were then labeled using mercury orange and from bulk tissue using high-performance liquid chromatography (HPLC). They also evaluated the abundance of cysteine, a rate limiting amino acid that is critical to the synthesis of GSH. The results of the study demonstrated that the low molecular weight thiols change distribution or are depleted upon APAP exposure in utero in general. Focusing on brain and brain precursor areas, these thiols are not uniformly distributed under baseline conditions to start with and in the neuroepithelium the levels tend to be low or variable in their presence, and upon treatment with APAP at the lowest dose (125 mg/kg) the thiols appeared to be almost absent altogether suggesting that the GSH was depleted (see Figure 1e-h in Beck et al., 2001). HPLC analysis of embryo tissue for GSH showed that there are drastic baseline developmental increases in GSH that were attenuated by 250 and 500 mg/kg APAP treatment (see Figure 3a), which corroborates the histological staining. These results show that APAP exposure in utero, at reasonable doses, is causing GSH depletion ostensibly via NAPQI detoxification, which, as discussed above, causes oxidative stress. Strengths of this study are the innovation in looking at brain GSH within the tissue microscopically and with an oral route of APAP administration, which is more comparable to typical APAP administration in humans. Some weaknesses of this study include that sex differences were not looked at or were not able to be evaluated, some of the doses were relatively high, and mercury orange histology might not be specific to GSH. This study was not included in the FDA's review of APAP's neurotoxic effects.

Parameter	**Scoring (Beck et al., 2001)**	**Score**
Direction of Effect	[APAP DNT (+) or not DNT (-)]	+
Controls	[Insufficient (0) Acceptable (1) Good (2)]	2
Sample Size	[Insufficient (0) Acceptable (1) Good (2)]	1
Outcomes	[Poor quality (0) Moderate Quality (1) Good Quality (3)]	1
Multi-Dose	[One dose (0) Multi dose (1)]	1
Blinding	[No blinding mentioned or conducted (0) Some Blinding (1) All "Blindable" Parameters Blinded (2)	0
Bias/Conflict Flag	Asterisk, does not alter score	**Total 5**

Dean et al., 2012 *European Journal of Neuroscience*. The purpose of this study was to examine the role of inflammatory prostaglandins in the early postnatal development of the cerebellum – a brain region that matures later in the postnatal period and one that is linked with social, learning and memory, and motor alterations in disorders including ASD. On postnatal day (P) P7 to P13 (equivalent to the third trimester of the human prenatal period), male and female Sprague-Dawley rats received daily subcutaneous injections of sesame oil (control) or one of three COX inhibitors: (1) nimesulide, a selective COX-2 inhibitor; (2) indomethacin, a non-selective COX-1 and COX-2 inhibitor; or (3) APAP, which inhibits prostaglandin production by a different mechanism The APAP group (*n*=5 males, 7 females) received 40 mg/kg body weight, which

corresponds to the human rectal APAP dose used in pediatric hospital settings. On P14, the spinophilin content in the cerebellar cortex was then evaluated using Western blot analysis. Spinophilin is a protein that is correlated with dendritic spines and corresponds to proper synaptic functioning. The APAP-treated rats had significantly increased cerebellar posterior vermis spinophilin (a protein associated with dendritic synaptic spines) compared to the control group indicating that developmental APAP administration induces cerebellar dendritic arborization and/or synaptic growth. Despite APAP being used as a control (used as a non NSAID prostaglandin inhibitor comparator to NSAIDs), this work nevertheless demonstrated that APAP and other prostaglandin inhibitors could act on developmental dynamics in the cerebellum, disrupting normal neurodevelopment. The authors used a combination of in vivo and in vitro approaches (NSAIDs only for the latter, however), and included both sexes but did not identify sex differences. The implication, however, is that APAP is capable of altering synaptic dynamics in the cerebellum – regions/damage that are both implicated in neurodevelopmental disorders.

This study's major limitation is that it was not designed to directly evaluate APAP for its potential as a neurodevelopmental toxicant and many/most of the additional analyses in the study did not include APAP. For this reason, I determined the outcomes of this study are only of moderate quality (1) for the purposes of this report. This study was not included in the FDA's review of APAP's neurotoxic effects.

Parameter	**Scoring (Dean et al., 2012)**	**Score**
Direction of Effect	[APAP DNT (+) or not DNT (-)]	+
Controls	[Insufficient (0) Acceptable (1) Good (2)]	2
Sample Size	[Insufficient (0) Acceptable (1) Good (2)]	2
Outcomes	[Poor quality (0) Moderate Quality (1) Good Quality (3)]	1
Multi-Dose	[One dose (0) Multi dose (1)]	0
Blinding	[No blinding mentioned or conducted (0) Some Blinding (1) All "Blindable" Parameters Blinded (2)	0
Bias/Conflict Flag	Asterisk, does not alter score	**Total 5**

Lichtensteiger et al., 2015 *Endocrinology*. In 2015, Lichtensteiger et al., published a study evaluating gene expression and behavior in rats exposed to APAP. In this study, APAP was administered by gavage to pregnant rats at 360 mg/kg/day from embryonic days E13-19 until weaning. Development and behavior were intact relative to control offspring, but gene expression profiling by quantitative real-time PCR (qRT-PCR) of hypothalamic brain areas showed significant alterations in a subset of key neurodevelopment genes including genes linked to ASD including Nrxn1, Nlgn1, Shank2, Dlg4, BDNF, CYP19A1, Nrg1, Erbb4, and Slit1. This study shows that gene expression is affected by APAP use in pregnant and perinatal mice. This study's use of gene expression as an outcome is a strength. However, more comprehensive set of genes, or all genes, would be better. This study was not included in the FDA's review of APAP's neurotoxic effects.

Parameter	Scoring (Lichtensteiger et al., 2015)	Score
Direction of Effect	[APAP DNT (+) or not DNT (-)]	+
Controls	[Insufficient (0) Acceptable (1) Good (2)]	2
Sample Size	[Insufficient (0) Acceptable (1) Good (2)]	2
Outcomes	[Poor quality (0) Moderate Quality (1) Good Quality (3)]	2
Multi-Dose	[One dose (0) Multi dose (1)]	0
Blinding	[No blinding mentioned or conducted (0) Some Blinding (1) All "Blindable" Parameters Blinded (2)	0
Bias/Conflict Flag	Asterisk, does not alter score	**Total 6**

Blecharz-Klin et al., 2015 *Environmental Toxicology and Pharmacology* [Blecharz-Klin et al., 2015a]. In 2015, Blecharz-Klin et al., equally divided pregnant rats into one of three groups: one control and two APAP-treated groups. The mothers had access to food and tap water (control) or APAP solution adjusted to a daily intake of 5 mg/kg (P5) or 15 mg/kg body weight (group P15) throughout pregnancy and lactation. On postnatal day 28, the male offspring were weaned and separated from the dams. They continued to receive APAP at doses of 5 (P5, *n*=9) or 15 mg/kg (P15, *n*=9) or tap water (Con, *n*=9) until they reached two months of age. The rats were euthanized by decapitation on postnatal day 60. Monoamines (neurotransmitters like serotonin that are characterized by a single amine group (-NH2) in its molecular structure), their metabolites and amino acid concentration in the medulla oblongata of male rats was measured using high-performance liquid chromatography (HPLC) with electrochemical detection.

Rats in the control group had the highest level of noradrenaline (NA) in the medulla oblongata; the P5 group had significantly less NA than the control group; and the P15 group had significantly lower levels of NA than both the control and P5 groups. Reductions in NA are noteworthy as this neurotransmitter has several important roles in neurodevelopment, and can also affect arousal, anxiety and fear, and in cognition. The P15 rats had a significant increase in noradrenaline metabolite MHPG compared to the control group. There were statistically significant differences in the MHPG/NA ratio between the P15 and control groups and between the P15 and P5 groups. The control group also had significantly higher dopamine concentration levels compared to P15 rats. The control group had significantly higher levels of homovanillic acid (HVA) compared to the P5 and P15 groups. HVA is a metabolite of dopamine, a critical neurotransmitter. The P15 group had significantly higher levels of serotonin (5-HT) than the P5 and control groups, and the ratio of 5-HT and its metabolite 5-hydroxyindoloacetic acid (5-HIAA) was decreased in the P15 group. P5 rats showed decreased concentrations of taurine and alanine compared to the control group. Blecharz-Klin and colleagues demonstrated that dopaminergic and noradrenergic neurotransmitter changes (abundance and metabolism) in the medulla oblongata of rats are affected by fetal and early life exposure to subtherapeutic levels of APAP. This finding is important because even transient disruption of neurotransmitters during development could have long-lasting neurodevelopmental impacts.

This study has several limitations, particularly the fact that the authors only looked at males and did not use functional data to link to the biochemical changes. Though a relatively low dose was used, they also continuously administered APAP until euthanasia at 60 days which extended

beyond the main window of vulnerability we are interested in, which makes it difficult to determine if the effects of APAP were transient or adaptive responses to compensate for neurocircuitry changes elsewhere in the brain. Accordingly, the results of this study are of poor to moderate quality for the purposes of this report. I assigned a score of 0.5. This study was not included in the FDA's review of APAP's neurotoxic effects.

Parameter	**Scoring (Blecharz-Klin et al., 2015a)**	**Score**
Direction of Effect	[APAP DNT (+) or not DNT (-)]	+
Controls	[Insufficient (0) Acceptable (1) Good (2)]	2
Sample Size	[Insufficient (0) Acceptable (1) Good (2)]	2
Outcomes	[Poor quality (0) Moderate Quality (1) Good Quality (3)]	0.5
Multi-Dose	[One dose (0) Multi dose (1)]	1
Blinding	[No blinding mentioned or conducted (0) Some Blinding (1) All "Blindable" Parameters Blinded (2)	0
Bias/Conflict Flag	Asterisk, does not alter score	**Total 5.5**

Blecharz-Klin et al., 2015 *International Journal of Developmental Neuroscience* [Blecharz-Klin et al., 2015b]. Blecharz-Klin et al., using an analogous design to the study above, looked at levels of neurotransmitters and amino acids in spinal cords of rat offspring of pregnant dams given tap water (control) or APAP solution adjusted to a daily intake of 5 mg/kg (group P5) or 15 mg/kg body weight (group P15) throughout pregnancy and lactation. On postnatal day 28, the male offspring were weaned and separated from the dams. They continued to receive APAP at doses of 5 (P5, n=9) or 15 mg/kg (P15, n=9) or tap water (Con, n=9) until they reached two months of age. At that point, the offspring were euthanized, and the spinal cord was prepared for analysis using HPLC with electrochemical detection. The concentration of monoamines, their metabolites and amino acids were measured. The spinal cords of P15 rats had significantly lower levels of MHPG than the control group. Dopamine concentration was significantly higher in the spinal cord of Control rats compared to the P5 and P15 groups; P5 group had significantly lower concentration of dopamine metabolite HVA compared to control; and the P15 group had significantly lower concentrations of dopamine metabolite 3-MT compared to control. There were no significant differences between the groups with respect to the spinal cord concentration of serotonin and its metabolites. The P15 rats had significantly increased concentration of aspartic acid in the spinal cord compared to the control and P5 groups, and although there were not significant differences between the groups with respect to the concentration of glutamic acid, analysis did show a tendency toward increased levels of glutamic acid in the P15 group compared to the control group. Blood analysis was used to measure liver and kidney function and showed no significant differences between groups with respect to the levels of alanine aminotransferase, aspartate aminotransferase, creatinine, serum alkaline phosphatase, γ-glutamyl transpeptidase, and creatine kinase (markers commonly used to evaluate liver and kidney health and function). This study demonstrates that exposure to low dose APAP prenatally and in early life affects neurotransmission and the concentration of amino acids in the spinal cords of male rat pups. As with their previous 2015 publication, the implication here is that developmental APAP exposure is altering neurochemistry, and this impact could alter neurodevelopment.

Limitations of this study include that the authors looked at males only, have no functional data to link to the biochemical changes, and continued administration of APAP until postnatal day 60. As with the previous study, this makes it difficult to determine if the effects of APAP were transient or adaptive responses to compensate for neurocircuitry changes elsewhere in the brain. Accordingly, the results of this study are of poor to moderate quality for the purposes of this report. I assigned a score of 0.5. This study was not included in the FDA's review of APAP's neurotoxic effects.

Parameter	**Scoring (Blecharz-Klin et al., 2015b)**	**Score**
Direction of Effect	[APAP DNT (+) or not DNT (-)]	+
Controls	[Insufficient (0) Acceptable (1) Good (2)]	2
Sample Size	[Insufficient (0) Acceptable (1) Good (2)]	2
Outcomes	[Poor quality (0) Moderate Quality (1) Good Quality (3)]	0.5
Multi-Dose	[One dose (0) Multi dose (1)]	1
Blinding	[No blinding mentioned or conducted (0) Some Blinding (1) All "Blindable" Parameters Blinded (2)	0
Bias/Conflict Flag	Asterisk, does not alter score	**Total 5.5**

Blecharz-Klin et al., 2016 *Pharmacological Reports*. In 2016, Blecharz-Klin et al., compared levels of neurotransmitters from the cerebellum from offspring of pregnant rats given control tap water (control) or APAP solution adjusted to a daily intake of 5 mg/kg (group P5) or 15 mg/kg body weight (group P15) throughout pregnancy and lactation. On postnatal day 28, the male offspring were weaned and separated from the dams. They continued to receive APAP at doses of 5 (P5, *n*=10) or 15 mg/kg (P15, *n*=10) or tap water (Con, *n*=10) until they reached two months of age. At that point, the rats were euthanized, and their cerebellum was prepared for analysis using HPLC with electrochemical detection. The concentration of monoamines, their metabolites and amino acids were measured. Rats in the P15 group had significantly higher cerebellar concentration of noradrenaline metabolite MHPG than control, and significantly higher MHPG/NA ratio than the P5 group. Research suggests that alterations in this ratio may reflect imbalances in the norepinephrine system, which could contribute to symptoms of neurodevelopmental disorder in humans (Ulke et al., 2019). The P5 group had a significantly higher concentration of 5-HIAA (a metabolite of serotonin) than the control group. There were no significant differences between groups with respect to dopamine and its metabolites. Alanine concentration was significantly higher in the P15 group compared to the P5 group. This study shows that being exposed to APAP during in utero and in early postnatal stages can cause a significant change in cerebellar neurotransmission, particularly the noradrenergic and serotonergic systems. As with their previous 2015 publications, the implication here is that developmental APAP exposure is altering neurochemistry and this could have implications for neurodevelopment. Some limitations of this study are that they looked at males only, did not collect functional data to link to the biochemical changes, and administered APAP until rats were two months old.

Parameter	Scoring (Blecharz-Klin et al., 2016)	Score
Direction of Effect	[APAP DNT (+) or not DNT (-)]	+
Controls	[Insufficient (0) Acceptable (1) Good (2)]	2
Sample Size	[Insufficient (0) Acceptable (1) Good (2)]	2
Outcomes	[Poor quality (0) Moderate Quality (1) Good Quality (3)]	0.5
Multi-Dose	[One dose (0) Multi dose (1)]	1
Blinding	[No blinding mentioned or conducted (0) Some Blinding (1) All "Blindable" Parameters Blinded (2)	0
Bias/Conflict Flag	Asterisk, does not alter score	**Total 5.5**

Blecharz-Klin et al., 2017 *Behavioural Brain Research*. In 2017, Blecharz-Klin et al., published another study evaluating the neurodevelopmental impact of low-dose APAP exposure on male rats. Female Wistar rats were mated and divided into three groups: control (received tap water), 5 mg/kg b.w. (P5) and 15 mg/kg b.w. (P15) groups who received an APAP solution in drinking water throughout pregnancy and lactation. At postnatal day 28 (PND28), male offspring were separated, and the control group (*Con*, *n*=10) continued receiving tap water, the P5 group (*n*=10) received 5 mg/kg APAP solution in drinking water, and the P15 group (*n*=10) received 15 mg/kg APAP solution in drinking water. On PND60, the rats were subjected to the following tests: (1) Staircase Test, to assess anxiety and exploratory/motor activity; (2) Hole Board Test, to estimate exploratory activity and locomotor skills; and (3) Water Maze Test (modified) to evaluate consolidation of spatial memory, visuomotor activity, motivation, and learning processes. There was no difference between control and APAP-treated groups on the Staircase Test. In the Hole Board Test, the P5 group had significantly lower motor activity and significantly fewer head dips than the Control and P15 groups; however, the number of climbings, crossings, and rearings was not impacted by APAP. The Water Maze Tests showed no significant differences between the groups during the spatial acquisition phase (days 1-4); in a probe trial the following day (day 5), the P5 group traveled across the area where the hidden platform had been significantly more than the Control group, and the P15 group had a reduced cumulative distance and mean swim velocity compared to the other groups; in the spatial reversal trials (days 8-9), the P5 group had significantly increased latency to the platform as compared to the P15 group; in the spatial reversal probe trial (day 10), the P5 group displayed a longer swim path and increased swimming velocity compared to the control, and demonstrated a preference for the reversal platform location and compared to the control and P15 groups, while the P15 group showed a lack of preference for the target quadrants and the control group preferred the original target quadrant; results of the visible platform test (day 11) indicated no differences in motor and visual capabilities between experimental groups.

Following the behavioral tests, the researchers euthanized the rats and prepared their prefrontal cortex, hippocampus, and striatum for biochemical analysis using HPLC. There were no statistically significant differences between the control and experimental groups with respect to noradrenaline and its metabolites in the brain structures. The rats exposed to APAP during neurodevelopment had elevated dopamine and DOPAC (a dopamine metabolite) and reduced HVA/DA and 3-MT/DA ratios (both biomarkers to assess the activity and balance of the dopamine system in the brain) in the striatum. The APAP-exposed groups also showed elevated 3-MT (a

dopamine metabolite) in the prefrontal cortex relative to the control group. There was a significant difference in the level of 5-HT (serotonin) in the prefrontal cortex of each group, and significantly higher levels of 5-HT in the prefrontal cortex and striatum of the P15 group. The APAP-treated groups also had increased 5-HIAA (a metabolite of serotonin) in the prefrontal cortex, and there was a significant decrease in the 5-HIAA/5-HT turnover in the striatum of APAP-treated rats, indicating altered serotonin function and signaling. They also found that the APAP-exposed rats had increased taurine, alanine, glutamic acid and histidine in the hippocampus, and increased aspartic acid decreased taurine concentration in the prefrontal cortex. The presence, levels, and proper balance of animo acids including those measured in this study are important for neurodevelopment processes including neuronal differentiation, synapse formation, synaptic plasticity, and cognitive function, and the disruption of the levels of animo acids in the hippocampus indicates disrupted neurodevelopment.

One limitation of the current study is that only males were used, which precluded comparison of biological sex differences. Additionally, as with other studies from this group, rats were also exposed to APAP until postnatal day 60. This study was not included in the FDA's review of APAP's neurotoxic effects.

Parameter	**Scoring (Blacharz-Klin et al., 2017)**	**Score**
Direction of Effect	[APAP DNT (+) or not DNT (-)]	+
Controls	[Insufficient (0) Acceptable (1) Good (2)]	2
Sample Size	[Insufficient (0) Acceptable (1) Good (2)]	2
Outcomes	[Poor quality (0) Moderate Quality (1) Good Quality (3)]	2.5
Multi-Dose	[One dose (0) Multi dose (1)]	1
Blinding	[No blinding mentioned or conducted (0) Some Blinding (1) All "Blindable" Parameters Blinded (2)	0
Bias/Conflict Flag	Asterisk, does not alter score	**Total 7.5**

Blecharz-Klin et al., 2018 *Pharmacology, Biochemistry and Behavior*. Blecharz-Klin et al., published a paper in 2018 as part of their series of studies in rats looking at prenatal and early life exposure to 5 or 15 mg/kg APAP exposure in male rats only. The study assessed cognitive and social behavior of rats and examined the BDNF levels in the pre-frontal cortex, hippocampus, and striatum. BDNF plays a key role in neurodevelopment, cognition, and neural plasticity. Pregnant rats received a solution of either 5 mg/kg or 15 mg/kg paracetamol via their drinking water per day. Control group rats were simply given water. APAP was also administered during lactation and after weaning through testing (PND 60). The authors found a nearly a two-fold decrease in BDNF levels in the striatum in APAP-treated rats relative to the control group. In social interaction testing, APAP-dosed rats showed significant alterations in multiple domains; reduced frequency of social interactions overall (figure reproduced from article, below), reductions in social sniffing behavior, and a significant increase in pinning behavior in the higher 15 mg/kg dose condition only compared to the control and P5 groups. The latter behavior is a normal play behavior, but it can be expressed excessively. Here, the authors interpret the behavior as excessive defensive behavior. There were no changes in the novel object recognition test associated with APAP treatment that would be interpretable as cognitive changes. Strengths of this study include the oral dosing route via the drinking water which is more analogous to oral acetaminophen use in

humans, and the use of the social behavior variables. Limitations of the study include the single-sex analysis, the lack of an effect on duration of social behaviors, and the continuation of APAP administration through PND 60. This study was not included in the FDA's review of APAP's neurotoxic effects.

Parameter	**Scoring (Blecharz-Klin et al., 2018)**	**Score**
Direction of Effect	[APAP DNT (+) or not DNT (-)]	+
Controls	[Insufficient (0) Acceptable (1) Good (2)]	2
Sample Size	[Insufficient (0) Acceptable (1) Good (2)]	1
Outcomes	[Poor quality (0) Moderate Quality (1) Good Quality (3)]	3
Multi-Dose	[One dose (0) Multi dose (1)]	1
Blinding	[No blinding mentioned or conducted (0) Some Blinding (1) All "Blindable" Parameters Blinded (2)	0
Bias/Conflict Flag	Asterisk, does not alter score	**Total 7**

Saeedan et al., 2018 *Inflammopharmacology*. In 2018, Saeedan et al. published "Effect of early natal supplementation of paracetamol on attenuation of exotoxin/endotoxin induced pyrexia and precipitation of autistic like features in albino rats." This study aimed to determine the underlying pathology beneath ASD. Rat pups were treated with various vaccines, APAP, the lipopolysaccharide (LPS) and combinations of the various drugs and chemicals and APAP. The animals were divided into eight groups consisting of four female rats placed in separate cages for delivering pups and treatment started just after the delivery of the pups. Groups were treated as follows:

Group I: received normal saline (3 mg/kg subcutaneously).
Group II: received APAP (50 mg/kg subcutaneously) on PND 5.
Group III: received the measles mumps rubella (MMR) (0.5 ml intraperitoneally).
Group IV: received the MMR (0.5 ml) with APAP (50 mg/kg subcutaneously).
Group V: received lipopolysaccharide (LPS) (50 µg/kg intraperitoneally).
Group VI: received the LPS (50 µg/kg i.p) with APAP (50 mg/kg subcutaneously.).
Group VII: received diphtheria tetanus and pertussis (DPT) (0.5 ml intraperitoneally).
Group VIII: received DPT (0.5 ml i.p.) with APAP (50 mg/ kg subcutaneously).

Postnatal growth and behavioral alterations were observed up to 45 days at different postnatal days depending on the test. Pups were evaluated for weight gain; eye opening; and behavior, including negative geotaxis test, swimming performance test, locomotion activity test, olfactory discrimination test (which was in essence a nest-seeking test), nociception (pain sensitivity), and elevated plus maze test. After behavioral testing, the animals were euthanized and whole brains were collected for biochemical assays. The assays included histopathology and brain tissue homogenates prepared for measuring acetylcholinesterase,[8] thiobarbituric acid reactive

[8] AChE is an enzyme that assists in the breakdown of acetylcholine, a neurotransmitter discussed previously in this report. In the context of neurodevelopment, AChE assists in regulating cholinergic signaling, impacting neuronal differentiation, synapse formation, and cognitive functions.

substances,[9] catalase, superoxide dismutase,[10] protein carbonyl,[11] and tissue glutathione and also evaluating interleukin levels, COX-1, COX-2 and 15-LOX.[12] APAP only exposed rats showed normal weight gain, but a significant delay in eye opening at postnatal day 17, relative to controls. They also showed hypoactivity and appeared to prefer new bedding as opposed to their home cage bedding in the nest-seeking test. This is in contrast to the control animals, who showed a strong preference for the soiled home cage bedding (the typical behavior for young rodent pups). Groups treated with APAP alone showed a significant decrease in locomotor activity compared to controls. There were significant alterations in cytokines and detoxification enzymes of the APAP group including in levels of SOD, catalase, protein carbonyls and GSH all indicative of oxidative stress.

The strengths of this paper are predominantly in the biochemical measurements. The behavioral data are present to validate the impact of the biochemical measurements, but the published version of the paper is missing details that make the results difficult to fully interpret, such as missing apparatus information for locomotor data, sex information, and information on proportion time in elevated plus maze arms instead of just the entries. This study was not included in the FDA's review of APAP's neurotoxic effects.

Parameter	**Scoring (Saeedan et al., 2018)**	**Score**
Direction of Effect	[APAP DNT (+) or not DNT (-)]	+
Controls	[Insufficient (0) Acceptable (1) Good (2)]	2
Sample Size	[Insufficient (0) Acceptable (1) Good (2)]	0.5
Outcomes	[Poor quality (0) Moderate Quality (1) Good Quality (3)]	0.5
Multi-Dose	[One dose (0) Multi dose (1)]	0
Blinding	[No blinding mentioned or conducted (0) Some Blinding (1) All "Blindable" Parameters Blinded (2)	0
Bias/Conflict Flag	Asterisk, does not alter score	**Total 3**

[9] Thiobarbituric Acid Reactive Substances (TBARS) are markers of oxidative stress and lipid peroxidation. Increased levels of TBARS generally indicate higher oxidative damage to lipids and cell membranes.

[10] Catalase and Superoxide Dismutase (SOD) are antioxidant enzymes which protect the body from oxidative stress.

[11] Protein carbonyl levels indicate protein oxidation, another marker of oxidative stress. In general, increased protein carbonyl content suggests oxidative damage to proteins.

[12] COX-1 and COX-2 are enzymes involved in the production of pro-inflammatory prostaglandins, while 15-LOX is an enzyme involved in the production of lipid mediators with anti-inflammatory properties. Evaluating the levels or activity of these enzymes provides insights into the inflammatory processes and lipid signaling involved in neurodevelopment.

Blecharz-Klin et al., 2019 *International Journal of Developmental Neuroscience*. In 2019, Blecharz-Klin and colleagues published a study focused on neurotransmitters and amino acids measured in rat hypothalamus as a function of prenatal and early life exposure to chronic low doses of APAP or water control. Pregnant female rats were exposed to 5 mg/kg or 15 mg/kg APAP via water depending on group. Male offspring were left with dams until 28 days of age and the pups were then given the solution through two months of age. Rats were then euthanized, and the hypothalamus was dissected from the brain for downstream analysis. The authors used high-performance liquid chromatography (HPLC) to estimate concentrations of monoamines, their metabolites, and amino acids. The control group had a significantly higher level of the main noradrenaline metabolite MHPG compared with both APAP exposure groups. The level of dopamine was significantly increased in rats treated with 5 mg/kg APAP, and there was a significant decrease of glutamic acid concentration in that group as well. The main dopamine metabolite HVA level was higher in both APAP exposed groups than the control. Increase in the concentration of the other dopamine metabolite DOPAC was also observed in the APAP treated groups compared with control. There were no statistically significant differences between groups in the 5-HT (serotonin)content or regarding the level of other amino acids.

The authors reported that the study showed that exposure to APAP prenatally and in initial postnatal life causes changes in the level of many neurotransmitters in the hypothalamus with the effect dependent on the dosage. The authors' stance is that their data are relevant for neurodevelopmental toxicity of APAP primarily as it has to do with the alterations in neurotransmission in the hypothalamus as a central regulator of common behavioral and developmental functions, as well as the hypothalamic-pituitary-gonadal axis which, when disturbed, could underlie hormonal, behavioral, and structural changes in reproductive and sexual disturbances that have been associated with developmental APAP exposure.

The strengths of this study are similar to the other papers reviewed in this report by the same research group. They use lower doses of APAP and covered a range of developmental windows that are likely relevant in terms of timing of exposure. However, this study from this group is limited in that there are no functional or behavioral readouts. In addition, most of the neurotransmitter changes are limited to single metabolite changes without alterations to the parent catecholamine or the ratio of the metabolite to the catecholamine. The exceptions are elevated dopamine (DA) in the 5 mg/kg group, and the glutamic acid finding. Taken together with the male-only design and continued administration of APAP up to postnatal day 60, the current study does have limitations. Nevertheless, the study does add to the evidence that there are statistically consistent changes in the brain associated with perinatal APAP exposure in rats at low doses. This study was not included in the FDA's review of APAP's neurotoxic effects.

Parameter	Scoring (Blecharz-Klin et al., 2019)	Score
Direction of Effect	[APAP DNT (+) or not DNT (-)]	+
Controls	[Insufficient (0) Acceptable (1) Good (2)]	2
Sample Size	[Insufficient (0) Acceptable (1) Good (2)]	1
Outcomes	[Poor quality (0) Moderate Quality (1) Good Quality (3)]	0.5
Multi-Dose	[One dose (0) Multi dose (1)]	1
Blinding	[No blinding mentioned or conducted (0) Some Blinding (1) All "Blindable" Parameters Blinded (2)	0
Bias/Conflict Flag	Asterisk, does not alter score	**Total 4.5**

Klein et al., 2020 *Neurotoxicology and Teratology*. Klein and colleagues evaluated whether APAP can induce developmental neurotoxicity in rats. Pregnant Wistar rats were gavaged with 350 mg/kg APAP (or water control) daily from day 6 of pregnancy until delivery. Behavior of male and female pups was evaluated at PND 10 for nest seeking behavior, PND 27 for apomorphine-induced behavioral stereotypy, PND 28 for sociability using the three-chamber sociability test and locomotion and emotionality using open field, and PND 29 for locomotion and emotionality using an elevated plus-maze and nociception using a hot plate. On PND 22, the study authors also evaluated lipid hydroperoxide (LOOH),[13] reduced glutathione (GSH), and brain-derived neurotrophic factor (BDNF) levels in the prefrontal cortex and hippocampus on another group of animals that did not undergo behavioral testing. There were no clinical signs of toxicity noted during gestation or lactation periods. The authors found that prenatal exposure to APAP impaired nest seeking behavior and augmented apomorphine-induced behavioral stereotypy.[14] APAP-exposed females showed elevated rearing behavior in open field test. The authors did not observe alterations in the LOOH, GSH, or BDNF levels.

The authors conclude that the results of the study point to behavioral changes relevant to neurodevelopmental disorders such as ASD and ADHD and that their results support that APAP is a developmental neurotoxicant. Strengths of the study include the number and types of behavioral studies used and the level of detail included in the publication's data tables in the publication. Some weaknesses of the study are that the dose is moderately high and the route of administration is oral gavage, which as discussed previously is somewhat less clinically relevant to human oral administration of APAP. However, any issues in terms of gavage causing the animals stress and potentially impacting results are mitigated by the appropriate controls. This study was not included in the FDA's review of APAP's neurotoxic effects.

[13] Lipid hydroperoxides (LOOH) are reactive compounds formed as a result of lipid peroxidation, and their presence and accumulation can contribute to oxidative stress.

[14] Behavioral stereotypy refers to repetitive, invariant behaviors that lack flexibility or variation.

Parameter	Scoring (Klein et al., 2020)	Score
Direction of Effect	[APAP DNT (+) or not DNT (-)]	+
Controls	[Insufficient (0) Acceptable (1) Good (2)]	2
Sample Size	[Insufficient (0) Acceptable (1) Good (2)]	1.5
Outcomes	[Poor quality (0) Moderate Quality (1) Good Quality (3)]	2.5
Multi-Dose	[One dose (0) Multi dose (1)]	0
Blinding	[No blinding mentioned or conducted (0) Some Blinding (1) All "Blindable" Parameters Blinded (2)	1
Bias/Conflict Flag	Asterisk, does not alter score	**Total 7**

Koehn et al., 2020 *F1000Research*. Koehn et al., (2020) examined the inflammatory response in the placenta and the fetal brain following maternal APAP exposure to determine if it was associated with alterations in placental and blood-brain barrier permeability. Pregnant rats were administered APAP by intraperitoneal injection in two groups. The first was administered APAP at a dose of 15 mg/kg once at embryonic day E19, replicating acute APAP exposure. The second was administered APAP twice per day at 3.75 mg/kg or 15 mg/kg for four days at E15-E19. To assess placental permeability, the study authors used a low-molecular weight, hydrophobic molecule sucrose to determine the transfer from the dams' circulation to the fetal circulation and from the fetal circulation back to the (anesthetized) dams. They also investigated the transfer of a large molecule, α-fetoprotein (AFP), across the placenta. To evaluate gene expression changes, they collected fetal brain tissue and placenta tissues and evaluated the expression of all genes using RNA-seq in acute or chronic 15 mg/kg vs control conditions (not 3.75 mg/kg). The authors found large numbers of genes that were altered in their expression using liberal statistics (p-value <0.05), and fewer but nevertheless numerous transcriptional changes in the APAP treated fetal brains.

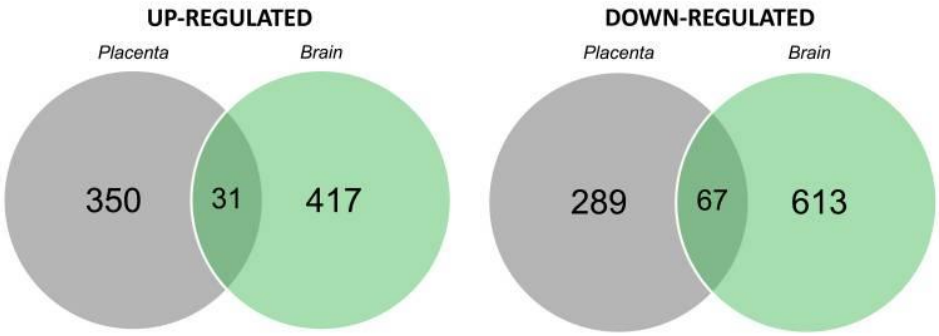


Figure 36. Number of up-regulated and down-regulated genes in the E19 placenta and brain following chronic maternal treatment of APAP.
Reproduced from Koehn et al., 2020.

Notably, there were more transcriptional effects in the tissues from the chronic APAP treated conditions as compared to the single dose condition. The main findings were strong evidence of inflammatory signaling in placenta that was largely not reflected in fetal brain tissue. Results from the placental permeability measures showed that placental transfer was potentially affected by APAP treatment, and demonstrated increased levels of AFP detected in blood plasma

of dams treated with APAP indicative of elevated fetal-to-maternal leakiness of placenta. There was no evidence of blood brain barrier damage by APAP treatment under these conditions. The impact of this research study is that APAP could elicit harm to the developing fetal brain via impacts to the placenta, which could then drive inflammatory signaling and damage to the fetal brain. As discussed earlier in this report, inflammation poses risk to the developing brain, and has a complex, bi-directional relationship with oxidative stress.

Strengths of this study included the innovative measures of placental (bidirectional) and brain permeability and powerful RNA-seq transcriptomics. Researchers used orthogonal measures of permeability which means they used complimentary but distinct ways to measure the effects of APAP. They displayed full length gels for AFP Western blots[15] which shows the rigor and transparency of the study, and the use of a non-pregnant control is a strength in the AFP Western blots and further validates the results.

Weaknesses of this study include that there were no sex-stratified analyses. RNA-seq significance is not adjusted p-value or FDR (false discovery rate) which limits the value of the findings, but authors do acknowledge fold change magnitude implications of their findings, suggesting that these findings still hold value despite the limitations in statistical significance. Generally, unadjusted p-value is not acceptable for calling significantly differentially expressed genes, but it can be acceptable for use in certain Gene Ontology (GO) term analyses, hypothesis generation, or rank based bioinformatics analyses. Some analyses are qualitative – e.g., permeability metrics and AFP Western Blot. This study was not included in the FDA's review of APAP's neurotoxic effects.

Parameter	**Scoring (Koehn et al., 2020)**	**Score**
Direction of Effect	[APAP DNT (+) or not DNT (-)]	+
Controls	[Insufficient (0) Acceptable (1) Good (2)]	2
Sample Size	[Insufficient (0) Acceptable (1) Good (2)]	1
Outcomes	[Poor quality (0) Moderate Quality (1) Good Quality (3)]	2.5
Multi-Dose	[One dose (0) Multi dose (1)]	1
Blinding	[No blinding mentioned or conducted (0) Some Blinding (1) All "Blindable" Parameters Blinded (2)	0.5
Bias/Conflict Flag	Asterisk, does not alter score	**Total 7**

Rigobello et al., 2021 *Behavioral Brain Research*. Rigobello and colleagues administered 35 or 350 mg/kg APAP (or water as a vehicle control) to pregnant mice starting at gestational day 6 (E6) to postnatal day 21 (weaning). They evaluated the offspring of both sexes among the three treatment conditions for behavioral outcomes as well as tissue level biochemical alterations. Behaviorally, they evaluated olfactory discrimination by looking at nest seeking behavior to see if

[15] AFP Western blots refer to Western blotting techniques used to detect and analyze Alpha-Fetoprotein (AFP) levels in biological samples. Western blotting, also known as immunoblotting, is a laboratory technique used to detect and analyze specific proteins in a biological sample.

the pups could detect and walk to bedding from their nest (while their eyes were still closed so they were blind). Once the pups were older, they measured motor activity and exploration in a (round) open field test, evaluated social interest and interactions in the three-chamber sociability task, and finally, evaluated susceptibility to pharmacologically-induced repetitive behavior. The Two-Way Analyses of Variance statistical tests used in this paper are acceptable and appropriate but can make the interpretation of the findings arduous.

There was no significant effect of APAP on nest seeking/olfactory behavior when the pups were 10 days old. Apomorphine induced stereotypy was statistically more pronounced in 350 mg/kg APAP exposed males, but not females, at latter timepoints tested but not the earlier timepoints tested. In the three-chamber sociability task, APAP treatment only affected transitions between compartments without exerting any notable effects on sociability measures. In the open field test, male rats developmentally exposed to the lower dose (35 mg/kg) of APAP showed enhanced activity in the peripheral part of the arena and females showed some treatment alterations in rearing and ambulation under some scenarios. The males in the lower dose of APAP also showed lower levels of reduced GSH in the hippocampus and reductions of SOD in the striatum but no other statistically significant biochemical changes were seen in other brain regions or for any regions for females at any APAP dose or for males at the higher 350 mg/kg dose.

Strengths of the study included the careful consideration of sex as a variable and multiple doses tested, well rationalized design, and inclusion of important biochemical markers including AOPP.[16] Weaknesses of the study include their factorial designs, which make it challenging to understand overall patterns of influence of the early life exposure to APAP on neurobehavioral phenotypes of the rats. Many of the statistical influences noted were via interactions that are challenging to contextualize, and the use of "marginal significance" is not sufficient for definitive research conclusions. This study was not included in the FDA's review of APAP's neurotoxic effects.

Parameter	**Scoring (Rigobello et al., 2021)**	**Score**
Direction of Effect	[APAP DNT (+) or not DNT (-)]	+
Controls	[Insufficient (0) Acceptable (1) Good (2)]	2
Sample Size	[Insufficient (0) Acceptable (1) Good (2)]	2
Outcomes	[Poor quality (0) Moderate Quality (1) Good Quality (3)]	2
Multi-Dose	[One dose (0) Multi dose (1)]	1
Blinding	[No blinding mentioned or conducted (0) Some Blinding (1) All "Blindable" Parameters Blinded (2)	1
Bias/Conflict Flag	Asterisk, does not alter score	**Total 8**

Suda et al., 2021 *PLoS ONE*. Suda and colleagues examined the interaction of maternal stress factors and repeated subcutaneous doses of a commercial APAP preparation OFIRMEV® in rat pups from postnatal days P4-P10 during postnatal development. The study included 85 rat pups

[16] AOPP are a group of modified proteins that are formed as a result of oxidative stress in the body.

from 14 litters who were divided into three treatment groups (two receiving the APAP preparation) and one control group. The pups injected with APAP (n=48) showed reduced rates of weight gain and elevated rates of "rearing behavior" which authors called a nonsocial behavior during a social play task when the rat offspring were studied at P37-P49. [My interpretation, given my training in the Blanchard Lab (https://neurotree.org/beta/publications.php?pid=5538), would be that elevated rearing behavior *could* reflect enhanced exploration, risk assessment (as non-social behaviors), or defensive social behavior. Those interpretations would require validation with orthogonal tests].

I scored the outcomes of this study as moderate in quality (1). One strength of this study is the use of a stressed background scenario that involved inducing maternal stress while the pups were in utero. The authors term this a "multi-dimension model" of the human experience. Weaknesses of the study include the lack of evaluation of sex differences and a lack of validation of non-social behavioral domains in the social play task.

While the authors include a non-treated control group, they did not have an APAP only condition to evaluate the difference between the commercial preparation of APAP containing mannitol and cysteine and that of just pure APAP. As a result, I consider the controls utilized in this study to be acceptable (1). This study was not included in the FDA's review of APAP's neurotoxic effects.

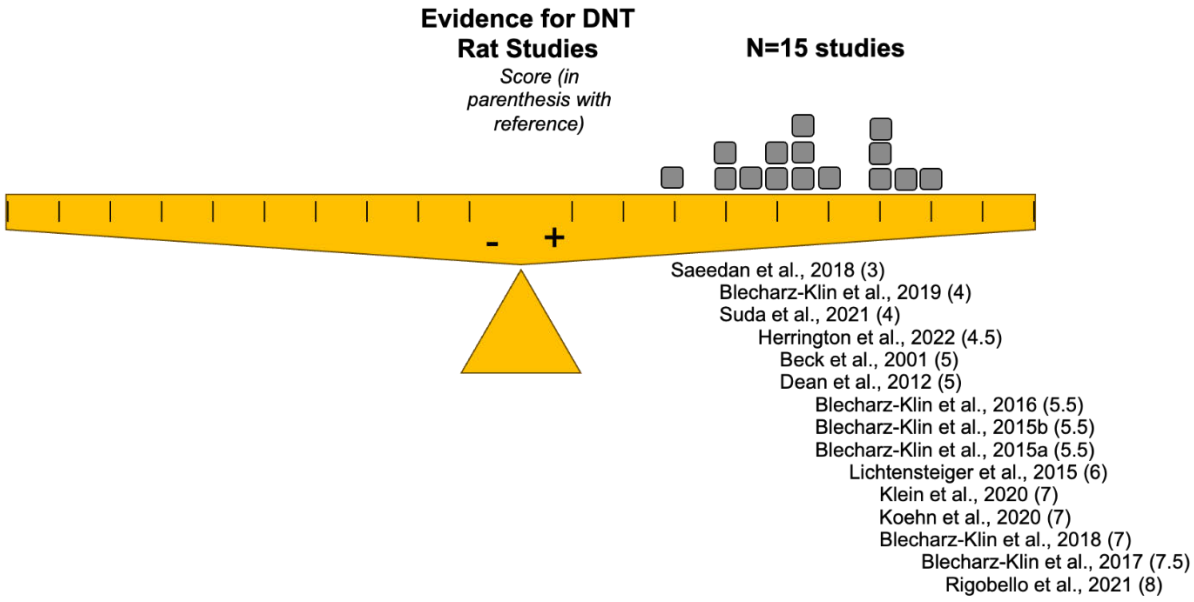
Parameter	**Scoring (Suda et al., 2021)**	**Score**
Direction of Effect	[APAP DNT (+) or not DNT (-)]	+
Controls	[Insufficient (0) Acceptable (1) Good (2)]	1
Sample Size	[Insufficient (0) Acceptable (1) Good (2)]	1
Outcomes	[Poor quality (0) Moderate Quality (1) Good Quality (3)]	1
Multi-Dose	[One dose (0) Multi dose (1)]	0
Blinding	[No blinding mentioned or conducted (0) Some Blinding (1) All "Blindable" Parameters Blinded (2)	1
Bias/Conflict Flag	Asterisk, does not alter score	**Total 4**

Herrington et al., 2022 *Developmental Psychobiology*. Herrington and colleagues sought to determine whether elevated ghrelin alters the behavioral effects of perinatal acetaminophen exposure in rats. Ghrelin is a neuropeptide, a small protein responsible for slow-onset, long-lasting modulation of synaptic transmission, shown to act as an antioxidant, reducing oxidative stress markers, and a neuroprotectant. The authors hypothesized that ghrelin administration prior to APAP exposure would mitigate certain effects of APAP exposure. 36 mixed sex rats were used from four litters. On PND 7, rats were exposed to one of four treatments: vehicle (saline), vehicle and ghrelin, 51.97 mg/kg APAP (the dosage was based on allometry to be equivalent to recommended dose for human children up to 5 years of age) and vehicle, 51.97 mg/kg APAP, and ghrelin via two subcutaneous injections given immediately after each other. On or about PND 60, the rats underwent the open field (OF) and Elevated Plus Maze (EPM) behavioral tests. In the OF test, rats treated with APAP exhibited much more exploratory behavior (higher proportion of center time) than controls while those treated with both APAP and ghrelin did not differ from controls. On the other hand, there was no significant effect of APAP exposure or significant interactions between APAP and ghrelin found in the EPM test. This difference in results of the

two tests could be partially due to the difference in length of the OF test (20 minutes) versus the EPM test (5 minutes). Statistical analyses do not indicate that the increased exploration of anxiolytic influence of the APAP only condition was driven by changes in locomotion.

A strength of this study is that treatments are distributed within litters which effectively controls for any litter effects which may be present. Display of the data as box and whisker plots with individual subject data is also a strength. Some weaknesses of this study include the route of administration is a subcutaneous injection (not as similar to oral administration in humans) and that the order of the OF and EPM was randomized. The experience of the rats in one arena affects their behavior in the other, so this should have been set and adhered to one way or the other. I would have performed OF first. Finally, the EPM variables of proportion time in the open arms, head dips, etc. are missing and are the most important variable from the EPM. Without them, we cannot fully evaluate anxiety-like behavior from this task in this study. This study was not included in the FDA's review of APAP's neurotoxic effects.

Parameter	**Scoring (Herrington et al., 2022)**	**Score**
Direction of Effect	[APAP DNT (+) or not DNT (-)]	+
Controls	[Insufficient (0) Acceptable (1) Good (2)]	2
Sample Size	[Insufficient (0) Acceptable (1) Good (2)]	1.5
Outcomes	[Poor quality (0) Moderate Quality (1) Good Quality (3)]	1
Multi-Dose	[One dose (0) Multi dose (1)]	0
Blinding	[No blinding mentioned or conducted (0) Some Blinding (1) All "Blindable" Parameters Blinded (2)	0
Bias/Conflict Flag	Asterisk, does not alter score	**Total 4.5**


Evidence for DNT
Rat Studies
Score (in parenthesis with reference)
N=15 studies
-
+
Saeedan et al., 2018 (3)
Blecharz-Klin et al., 2019 (4)
Suda et al., 2021 (4)
Herrington et al., 2022 (4.5)
Beck et al., 2001 (5)
Dean et al., 2012 (5)
Blecharz-Klin et al., 2016 (5.5)
Blecharz-Klin et al., 2015b (5.5)
Blecharz-Klin et al., 2015a (5.5)
Lichtensteiger et al., 2015 (6)
Klein et al., 2020 (7)
Koehn et al., 2020 (7)
Blecharz-Klin et al., 2018 (7)
Blecharz-Klin et al., 2017 (7.5)
Rigobello et al., 2021 (8)

2. *Mouse Studies*

Year	Author(s)	Strain/ Stock	Sex	Exposure Route	APAP Dose mg/kg/day	Score	In FDA review?
2014	Viberg et al.	NMRI	Male	Subcutaneous injection	0, 30, 60	5.5	Yes
2016	Saad et al.	Swiss CD-1	Both	Gavage	0, 150	-8.5*	Yes
2017	Hay-Schmidt et al.	C57BL/6J	Male	Gavage	0, 50, 150	7.5	No
2017	Philippot et al.	NMRI	Both	Subcutaneous injection	0, 60	5.5	Yes
2018	Philippot et al.	NMRI	Male	Subcutaneous injection	0, 30, 60	6.5	No
2021	Philippot et al.	NMRI	Male	Subcutaneous injection	0, 60	-3.5	No
2022	Philippot et al.	NMRI	Male	Subcutaneous injection	0, 60	6.5	No
2022	Harshaw &Warner	C57BL/6J	Both	Subcutaneous injection	0, 103.9	9	No
2023	Baker et al.	C57BL/6J	Both	Oral	0, 150	7.5	No

Viberg et al., 2014 *Toxicological Sciences*. In this study, male mice received a subcutaneous injection of saline, 30 mg APAP/kg, or two 30 mg APAP/kg doses four hours apart on postnatal day 10. Six male mice from all three groups were euthanized one hour after injection, and six male mice from the single dose group were also euthanized at 2- and 4-hours post-injection. Researchers used high-performance liquid chromatography with electrochemical detection (HPLC-EC) to determine APAP concentration in the neonatal mouse brains. They found that 96.5% of APAP was metabolized during the 1-hour to 2-hour post-injection timeframe, and mice who received repeated APAP doses (*i.e.*, 2 injections of 30 mg APAP/kg 4 hours apart) had substantially similar concentrations of APAP in the brain 1 hour after their second injection. Nine male mice from the control group and the repeated treatment group were euthanized at 24 hours after injection and their frontal cortex, parietal cortex, and hippocampus were collected and the amount of brain-derived neurotrophic factor (BDNF) was measured. Researchers found that the mice who received repeated doses of APAP had a 183% increase in BDNF in the fontal cortex and a 26% decrease in BDNF in the parietal cortex compared to the control mice. There was no significant difference in BDNF in the hippocampus of control or repeated APAP dose mice.

At 4-5 weeks of age, the male mice were separated and placed in groups of 4-7 and, at 2 months of age behavioral testing began.

- Spontaneous behavior testing showed that the activity of control and single APAP dose mice decreased throughout the 60-minute period they spent in the novel home environment. This is normal in control mice, as it indicates habituation to a new environment. Repeated dose mice showed significantly decreased activity during the first 20 minutes and increased activity in the last 20 minutes they spent in the novel home environment, indicating that repeated neonatal APAP exposure reduced habituation and increased hyperactivity, or an overall lack of habituation.
- One week later, 8 mice from each group were tested on the radial arm maze on three consecutive days. Mice in the control and single dose groups showed a significant decrease in errors and time to complete the task across the 3 days. The repeated dose group had significantly more errors and took substantially more time to complete the task than the control group on days 2 and 3, demonstrating a reduced spatial working memory.
- The next week, mice from each of the three groups were received an injection of saline or 30 mg APAP/kg thirty minutes prior to the Hot Plate Test. Mice who received saline pretreatment showed no significant differences in time on the hot plate regardless of neonatal APAP exposure. Among animals who received APAP treatment prior to testing, those who had neonatal APAP exposure (single or repeated) spent significantly less time on the hot plate than mice who had no neonatal APAP exposure, and mice who had repeated neonatal APAP exposure spent significantly less time on the hot plate than those who had a single dose. This indicates that neonatal APAP exposure does not impact pain sensitivity in adult mice; however, there is a neonatal dose response-related decrease in the analgesic/antinociceptive effect of APAP in adult mice.
- One week later, mice from each of the three groups were pretreated with saline or 30 mg APAP/kg and their behavior was analyzed in the Elevated Plus Maze. Animals who were pretreated with saline showed no differences between the control group and the neonatal APAP exposure groups. Among the mice who received APAP pre-treatment, those who received a single dose of neonatal APAP spent significantly less time in the open arms than the control group, and the mice who had repeated APAP doses made significantly fewer entries and spent significantly less time in the open arms than the control group. This indicates that neonatal APAP exposure did not affect anxiety-like behavior in adults, but it does change adults' anxiolytic response to APAP.

Some strengths of this study are the strong justification for the developmental timepoint tested, the relatively low dose tested, blinding to experimental condition in behavioral testing and measurement of pup brain APAP levels. Additionally, the measurement of BDNF, a molecule important in behavior and neuroplasticity is a strong attribute. Some weaknesses are male only sex, route of administration (subcutaneous) and the random selection of mice from cages for behavioral testing without accounting for prior experience. Ideally all animals would have experienced the same thing or “fresh” mice would be used for each new test every week which would require individual identification of pups which could have been accomplished with tattoos or ear notching.

Parameter	Scoring (Viberg et al., 2014)	Score
Direction of Effect	[APAP DNT (+) or not DNT (-)]	+
Controls	[Insufficient (0) Acceptable (1) Good (2)]	0.5[#]
Sample Size	[Insufficient (0) Acceptable (1) Good (2)]	1
Outcomes	[Poor quality (0) Moderate Quality (1) Good Quality (3)]	2
Multi-Dose	[One dose (0) Multi dose (1)]	1
Blinding	[No blinding mentioned or conducted (0) Some Blinding (1) All "Blindable" Parameters Blinded (2)	1
Bias/Conflict Flag	Asterisk, does not alter score	**Total 5.5**
[#]*Because of the way mice were selected from the cages for weekly testing, the comparators become complicated.*		

Saad et al., 2016 *PLoS One*. Saad et al. sought to determine whether in utero exposure to APAP caused hyperkinetic dysfunction in offspring using a mouse model. Eight pregnant CD1 mice were dosed with 150 mg/kg APAP each day from embryonic day 7 through delivery (day 21); eight pregnant mice in the control group were dosed with the 0.5% carboxymethylcellulose vehicle during the same time frame. After weaning, two males and two females from each litter were randomly selected for locomotor testing. Mice were placed in a plexiglass cage for two hours/day over three days and their locomotor activity (*i.e.*, vertical activity ("rearing"), spending time in the central versus peripheral area of the enclosure, fine repetitive movements, total ambulation, and total locomotor activity) was measured. The researchers found no significant behavioral differences between the APAP-exposed and control groups using their strict alpha level cutoff ($p<0.008$ instead of the customary $p<0.05$) for the number of comparisons. I have major issues with this and expand on this more below. Following the locomotor testing, the offspring were euthanized and their brains were processed for imaging. MRI was performed and 29 areas of the brain were evaluated. The authors found no significant differences in brain volume in those 29 areas between control and APAP-exposed mice. The brains were then prepared for neuronal quantification using Nissl staining (an imaging technique that stains or changes the color of molecules of DNA and RNA), which showed no significant difference in cortical neuron density between control and APAP-exposed offspring.

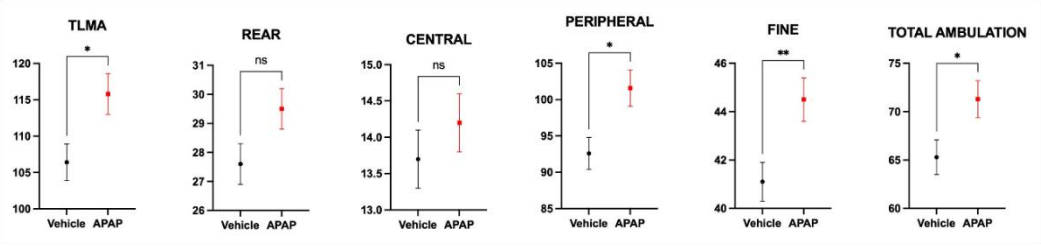
However, Saad et al.'s conclusion that "[f]indings from this study do not support a causal relationship" "between maternal exposure to APAP and risk of ADHD in mouse offspring" is the result of unnecessary statistical corrections. First, the authors unnecessarily adjusted the P value to 0.008 for statistical significance. When analyzed at the customary probability value of $p<0.05$, Saad and colleagues' findings are actually quite significant. For example, Saad and colleagues did find **a significant effect of APAP on hyperactivity** in their adjusted model (Table 1, second row reports an overall adjusted risk ratio for "Total Ambulation" of 1.181 with a 95% confidence interval of 1.007-1.385 and a p-value of 0.041). The separate adjusted models for males and female (though neither were significant given small samples sizes), shows a higher incidence rate ratio for male mice compared to female mice which harmonizes with my own work in mice (Baker et al., 2023).

Second, the authors unnecessarily employed an overly complicated multivariate statistical model to control for mouse offspring weight, gender, random selection, and testing time and day.

Typically, a covariate approach is well rationalized due to a complex ecological or clinical-type study design. But in this study, there is no clear justification for controlling for mouse weight unless they show that the weights were significantly different among treatment groups. In other words, if there is no reason for a variable to be confounding the data then it should not be included in a multivariate model. This technique only serves to reduce the power to see a difference in the groups.

Saad et al. refused to describe the changes they observed in motor behavior as significant despite having already showed to be significantly different in their adjusted model in Table 1. Their refusal appears to be based on their adjustments for multiple testing.

To highlight the extent to which the behavioral data are striking and meaningful in the Saad et al., study, I plotted their summary data with simple means comparisons (unpaired t-tests) and the resulting significant findings for total locomotor activity (TLMA) ($p<0.05$), Peripheral Activity, Fine Motor Activity ($p<0.01$), and Total Ambulation. Despite the significant incidence rate ratio in Table 1 and the clear effects in Table 2, the authors nevertheless claim in the discussion "... our behavioral studies indicate no differences in locomotor activity between the two groups." I do not know how this made it through peer review.



GROUP ASSIGNMENT	TLMA	Rear	Central	Peripheral	Fine	Total Ambulation
CONTROL	106.4 ± 2.5	27.6 ± 0.7	13.7 ± 0.4	92.6 ± 2.2	41.1 ± 0.8	65.3 ± 1.8
ACETAMINOPHEN	115.8 ± 2.8	29.5 ± 0.7	14.2 ± 0.4	101.6 ± 2.5	44.5 ± 0.9	71.3 ± 1.9

TLMA, total locomotor activity was calculated from addition of central and periphery gross motor activity and fine activity. N = 16/group. Data are presented as the mean ± s.e.m. Unis are number of events.

doi:10.1371/journal.pone.0157380.t002

Table 2. Mean values of different activities according to group assignment.

Figure 37. Analysis conducted using GraphPad Prism 9, two-tailed test, data present Mean ± 1 Standard Error of the Mean. Table modified from Saad et al., 2016.

Initially, it seemed that Dr. Saad and colleagues may customarily do Bonferroni-type multiple comparison corrections for their alpha levels in their similar mouse in vivo experiments. If that were the case, I could easily just say that this is just an over-rigorous group that is not afraid of Type II errors in order to avoid Type I errors (essentially, their group would prefer to have over-cautious statistics that err on the side of saying there is not an effect when there is, as opposed to saying there is an effect, when in fact, there is not). In general, it is good to err on the side of Type II errors, but not when it comes to where the safety of a drug is concerned. Instead, I would be more open to avoiding Type II error in support of safety and precaution, particularly in the context of an extremely vulnerable population. Various strong and well supported academic and

statistical points have been made in support against this widespread adjustment of p-value thresholds through division (e.g., Althouse, 2016; Feise, 2002; Rubin, 2021).

In an attempt to understand Saad et al.'s rationale in this study, I looked at other publications by Dr. Saad from around the same time to see if the same or analogous kind of harsh multiple correction testing was being performed as it was in this APAP paper. I reviewed Dr. Saad's academic site (https://researchexperts.utmb.edu/en/persons/antonio-saad, accessed 5/25/23, screenshot below) to review publications that might employ similar approaches and that would be approximately contemporaneous. I focused on the two immediately before and after the APAP paper.

High-fructose diet in pregnancy leads to fetal programming of hypertension, insulin resistance, and obesity in adult offspring
Saad, A. F., Dickerson, J., Kechichian, T. B., Yin, H., Gamble, P., Salazar, A., Patrikeev, I., Motamedi, M., Saade, G. R. & Costantine, M. M., Feb 15 2016, (Accepted/In press) In: American Journal of Obstetrics and Gynecology.
Research output: Contribution to journal › Article › peer-review
Fructose
Fetal Development
Insulin Resistance
Obesity
Diet
66
Scopus citations
175
Is There a causal relation between maternal acetaminophen administration and ADHD?
Saad, A., Hegde, S., Kechichian, T., Gamble, P., Rahman, M., Stutz, S. J., Anastasio, N. C., Alshehri, W., Lei, J., Mori, S., Kajs, B., Cunningham, K. A., Saade, G., Burd, I. & Costantine, M., Jun 2016, In: PloS one. 11, 6, e0157380.
Research output: Contribution to journal › Article › peer-review
Open Access
Acetaminophen
Attention Deficit Disorder With Hyperactivity
Mothers
Locomotion
Alanine Transaminase
8
Scopus citations
4
Maternal Fructose Consumption Disrupts Brain Development of Offspring in a Murine Model of Autism Spectrum Disorder
Saad, A., Alshehri, W., Lei, J., Kechichian, T. B., Gamble, P., Alhejaily, N., Shabi, Y., Saade, G., Costantine, M. & Burd, I., Mar 16 2016, (Accepted/In press) In: American Journal of Perinatology.
Research output: Contribution to journal › Article › peer-review
Fructose
Autism Spectrum Disorder
Contactins
Putamen
Mothers
2
Scopus citations
8

In the first paper with the title beginning "High-fructose diet...", the authors state:

> "All measurements were obtained blinded as to group assignment. Analysis was performed using software (STATA 14.0; StataCorp, Dallas, TX). Kolmogorov-Smirnov test was used to check for normal distribution of the data. Data are reported as mean ± SEM or median with interquartile range as appropriate. Statistical analysis was performed using an unpaired Student *t* test or Mann-Whitney test for nonparametric data. For the intraperitoneal glucose tolerance testing a 2-way repeated measures analysis of variance with Bonferroni correction was used. We did not correct for multiple testing since this is an exploratory study. **A *P* value <.05 was considered statistically significant.**"

In the second paper: "Maternal Fructose...", Saad and colleagues performed brain imaging and volumetric quantification of 29 brain regions in mice of dams exposed to fructose or control conditions. In this case, they did not correct for multiple comparisons, and as written in their methods

"Analysis was performed using STATA (StataCorp 14.0, Dallas, TX). Individual brain areas volumes were normal to brain volumes. Data are reported as mean standard error of the mean. Statistical analysis was performed using t-test or a Mann–Whitney U test as appropriate. **A p-value less than 0.05 was considered statistically significant.**"

My assessment from these two papers in mice from the same general period is that correction of alpha levels was not universally performed by this group and may have been used injudiciously when it served a purpose rather than for statistical rigor.

As a result of the shortcomings described above, I do not share the authors' conclusions. **I consider Saad et al., 2016 to be causal preclinical evidence of neurodevelopmental impacts of prenatal APAP effects on neurobehavioral outcomes such as hyperkinetic disorders and ADHD.** That my conclusion conflicts with the study's authors is indicated by an asterisk (*) next to the total score. Nevertheless, the direction of effect is given as negative to adhere to the conclusions of the peer reviewed publication, in order to be objective.

Parameter	**Scoring (Saad et al., 2016)**	**Score**
Direction of Effect	[APAP DNT (+) or not DNT (-)]	-
Controls	[Insufficient (0) Acceptable (1) Good (2)]	2
Sample Size	[Insufficient (0) Acceptable (1) Good (2)]	1.5
Outcomes	[Poor quality (0) Moderate Quality (1) Good Quality (3)]	3
Multi-Dose	[One dose (0) Multi dose (1)]	0
Blinding	[No blinding mentioned or conducted (0) Some Blinding (1) All "Blindable" Parameters Blinded (2)	2
Bias/Conflict Flag	Asterisk, does not alter score	**Total -8.5***

Philippot et al., 2017 *Journal of Applied Toxicology*. Philippot et al. demonstrated that exposure to clinically relevant doses of paracetamol (APAP) on postnatal day (PND) 3 and 10, but not 19, affected spontaneous behavior and habituation in adult mice aged 2 months when compared to the control group. The critical period between PND 3 and 10 in mice is comparable to the beginning of the third trimester to the time around birth in humans. Exposure on PND 19 would correspond to postnatal exposure in humans, somewhere comparable to late infancy or toddler period. Mice in this study were administered two subcutaneous injections of APAP at a dose of 30 mg/kg four hours apart. On PND 56, spontaneous activity, including locomotion, rearing, and total activity, was measured over 60 minutes in an automated device. This was done with ten mice randomly selected from three to five litters. Control mice showed normal habituation, defined by a time-dependent decrease in activity over the spontaneous behavior recording. Male mice exposed to APAP on PND 3 and 10 displayed significantly decreased activity compared to their controls during the first 20 minutes of testing for spontaneous behavior and a significant increase in activity compared to their controls in the last 20 minutes of testing. In these mice, there was a greater percentage of disruption of habituation in rearing compared to locomotion. Male mice exposed to APAP on PND 19 did not show significant changes in spontaneous behaviors compared to controls, showing that the mice were more susceptible to behavioral changes from APAP use when exposed during period more comparable to in utero brain development in humans (as opposed to the late infancy period). Female mice in the experiment

showed similar results. The study authors found no significant difference between male and female habituation quotient for locomotion or rearing. The authors indicated that their study showed that "adverse effects on adult behavior and cognitive function occurred in both male and female mice exposed to paracetamol" on those postnatal days which correspond to the beginning of the third trimester and the time of birth in humans. The authors stated that the dose they used was clinically relevant based on the body surface area normalization method. These results were not found in mice administered APAP at PND 19.

Strengths of this study include the inclusion of male and female mice, and low doses of APAP which are clinically relevant to human APAP use. Weaknesses of this study include the lack of statistical comparisons for rearing and the rather narrow focus on habituation for locomotion. Blinding was not mentioned.

Parameter	**Scoring (Philippot al., 2017)**	**Score**
Direction of Effect	[APAP DNT (+) or not DNT (-)]	+
Controls	[Insufficient (0) Acceptable (1) Good (2)]	2
Sample Size	[Insufficient (0) Acceptable (1) Good (2)]	2.5
Outcomes	[Poor quality (0) Moderate Quality (1) Good Quality (3)]	1
Multi-Dose	[One dose (0) Multi dose (1)]	0
Blinding	[No blinding mentioned or conducted (0) Some Blinding (1) All "Blindable" Parameters Blinded (2)	0
Bias/Conflict Flag	Asterisk, does not alter score	**Total 5.5**

Hay-Schmidt et al., 2017 *Reproduction*, Hay-Schmidt et al. focused on the effect of APAP and aniline (an organic aromatic amine and precursor that can be used to synthesize acetaminophen) on intrauterine brain development, specifically investigating urinary behavior, aggression, and sexual behavior in mice following administration of APAP or aniline (disregarded from further discussion because of its lack of relevance) to pregnant mice. Pregnant dams were treated with either water, 50 mg/kg/day APAP or 150 mg/kg/day APAP, from post-coitum (embryonic) day 7 to delivery via oral gavage. The authors stated that according to the estimate of mouse dose based on allometry, the doses used were well below APAP doses used by pregnant women of 50 mg/kg/day. The authors found that exposure to the highest dose of APAP resulted in significantly decreased cell number in the sexually dimorphic nucleus (SDN) in the preoptic area (POA) in the anterior hypothalamus of male adult offspring. Decrease in neuronal number in the SDN-POA is associated with reductions in male-typical behavior. Consistent with this, the authors found that exposure to the highest dose of APAP in utero showed changes in urinary scent marking behavior as adults, less aggressive territorial display and aggression, and reduced intromissions during mating.

While I generally excluded papers that focused on sexual behaviors and reproduction from my analysis, this paper showed direct alterations in cellular architecture in the brains of offspring mice exposed to APAP. Because of these alterations, I included it in this report's analysis. Some strengths of this paper include the oral route of APAP administration, full blinding, multiple doses tested, appropriate tests of aggression and sex typical behavior. Weaknesses include the focus only

on male behaviors and brain cellular architecture, and an acknowledged lack of insight into the mechanism as to the cellular change itself and its role (if any) in the observed behavioral changes.

Parameter	Scoring (Hay-Schmidt et al., 2017)	Score
Direction of Effect	[APAP DNT (+) or not DNT (-)]	+
Controls	[Insufficient (0) Acceptable (1) Good (2)]	2
Sample Size	[Insufficient (0) Acceptable (1) Good (2)]	1
Outcomes	[Poor quality (0) Moderate Quality (1) Good Quality (3)]	1.5
Multi-Dose	[One dose (0) Multi dose (1)]	1
Blinding	[No blinding mentioned or conducted (0) Some Blinding (1) All "Blindable" Parameters Blinded (2)	2
Bias/Conflict Flag	Asterisk, does not alter score	**Total 7.5**

Philippot et al. 2018 *Toxicological Sciences*. Philippot et al. sought to clarify the potential role of the CB1R (Cannabinoid Receptor Type 1, discussed previously in this report) in APAP-induced developmental neurotoxicity. Male mice were exposed on PND 10 to APAP, CB1R agonist WIN 55 212-2 (WIN), or various combinations of APAP and WIN via subcutaneous injection(s), while controls were injected with saline. Mice exposed to 2 doses of APAP, 4 hours apart displayed locomotor, rearing, and total activity levels that were significantly different from the controls. Specifically, with respect to APAP exposure, the study demonstrated that mice neonatally exposed on PND 10 to 2 doses of APAP (30 + 30 mg/kg) 4 hours apart displayed activity levels that were significantly different from the controls. Locomotor, rearing and total activity levels of the exposed mice were reduced during the first 20 mins of testing and increased during the second and third 20 min periods of testing compared to controls – a pattern noted in numerous of this research group's other publications with APAP in this novel cage habituation/locomotor task.

Mice exposed to a combination of AAP and WIN had significantly different activity levels of control animals and those exposed to a single dose of APAP or WIN. And mice exposed to repeated AAP doses together with a single dose of WIN showed altered spontaneous behavior compared with all other exposure groups. The authors also found that WIN and APAP exposure in a combination decreased cerebral cortical transcript levels of endocannabinoid-associated enzyme FAAH, an enzyme essential in endocannabinoid signaling, but did not find the same effect with exposure to AAP and WIN alone. No protein level changes associated with APAP or WIN exposures were detected. The authors concluded that "there was a more severe AAP-induced neurotoxic effect in the adult mouse following neonatal co-exposure to the CB1R agonist WIN. This extends our knowledge on (1) the delicate role of the CB1R during the BGS [brain growth spurt] and (2) its potential role in the developmental neurotoxicity of A[P]AP." [I added contents in brackets for clarity].

Some strengths of this study include that the expression analysis using quantitative PCR (qPCR) was well powered and sound research technique for mechanistic studies. Weaknesses of this study include that only males were used, the subcutaneous injection route of injection has some limitations in terms of matching human use of APAP. Additionally, there may be more comprehensive or sophisticated ways of determining if CB1R is involved in the neurobehavioral

effects of postnatal day 10 APAP exposure, for instance, by using a knockout mouse (Klinger-Gratz et al., 2018), or even an antagonist or inverse agonist (e.g., AM251) to block the effect rather than looking for additive influences. This study was not included in the FDA's review of APAP's neurotoxic effects.

Parameter	**Scoring (Philippot al., 2018)**	**Score**
Direction of Effect	[APAP DNT (+) or not DNT (-)]	+
Controls	[Insufficient (0) Acceptable (1) Good (2)]	2
Sample Size	[Insufficient (0) Acceptable (1) Good (2)]	2.5
Outcomes	[Poor quality (0) Moderate Quality (1) Good Quality (3)]	2
Multi-Dose	[One dose (0) Multi dose (1)]	0
Blinding	[No blinding mentioned or conducted (0) Some Blinding (1) All "Blindable" Parameters Blinded (2)	0
Bias/Conflict Flag	Asterisk, does not alter score	**Total 6.5**

Philippot et al., 2021 *International Journal of Developmental Neuroscience*. This study sought to determine whether neonatal (PND 10) exposure to APAP in mice affected their dentate gyrus (DG) thickness, as generation of DG cells is important to memory processing. Approximately 85% of DG neurons are generated in the first two postnatal weeks in rodents and synaptogenesis reaches adult levels at postnatal day 25. The authors used a single-day exposure model in an attempt to correspond their results more translationally to humans and to extend their previous research with APAP and endocannabinoids. The authors performed two experiments. In one, NMRI mice pups were subcutaneously injected with either two doses of 30 mg/kg APAP four hours apart or saline on PND 10. Three male mice (one each from three litters) from each exposure group were euthanized for study of the morphometry of the adult DG using staining of free-floating sections of the brains. The second experiment looked at gene transcript levels of markers for cell migration and neurogenesis. This experiment utilized male pups from four different litters also administered either two doses of 30 mg/kg APAP four hours apart or saline control. These mice were euthanized 24 hours after exposure, following which the hippocampus was obtained and evaluated. There were no signs of toxicity observed in either experiment.

Brains from three males each per condition (vehicle or APAP) were evaluated by measuring dentate gyrus size (length, width, and thickness). By using effect sizes and confidence intervals authors state that there are suggestions of differences but none that can be claimed as robust given the overlap of the confidence intervals with zero. The authors did not perform standard statistical means comparisons (unpaired t-tests). I ran their data using unpaired t-tests and indeed none are statistically significant (p-values ranging from 0.27-0.86). Likewise, their expression analyses showed no statistically significant transcriptional changes. Accordingly, either P10 APAP exposure does not affect these markers, or their study was underpowered to show such an effect. Strengths of this study include clever use of novel markers. Weaknesses of this study include low sample sizes, a route of administration that is uncommon in people, and the use of male mice only. This study was not included in the FDA's review of APAP's neurotoxic effects.

Parameter	Scoring (Philippot al., 2021)	Score
Direction of Effect	[APAP DNT (+) or not DNT (-)]	-
Controls	[Insufficient (0) Acceptable (1) Good (2)]	2
Sample Size	[Insufficient (0) Acceptable (1) Good (2)]	0.5
Outcomes	[Poor quality (0) Moderate Quality (1) Good Quality (3)]	1
Multi-Dose	[One dose (0) Multi dose (1)]	0
Blinding	[No blinding mentioned or conducted (0) Some Blinding (1) All "Blindable" Parameters Blinded (2)	0
Bias/Conflict Flag	Asterisk, does not alter score	**Total –3.5**

Philippot et al., 2022 *Frontiers in Toxicology*. Philippot and colleagues sought to evaluate how clinically relevant dosing of APAP in mice affects neurobehavioral and neurochemical outcomes. They administered two 30 mg/kg APAP (or saline control) injections subcutaneously to postnatal day 10 mice 4 hours apart on the same day. After weaning, mouse pups were assessed for spontaneous activity and habituation behavior when placed in a new clean cage. Statistical results showed an interaction between time and treatment such that at the latter time point, APAP (labeled AAP in this study) treated mice showed more locomotion indicative of hyperactivity or decreased habituation (the author's interpretation). See the figure from the paper, below. The top panel marked "A" displays these data. To test learning, memory and cognitive flexibility the mice were assessed in the Morris Water Maze (MWM). During training (learning) phases, the APAP treated mice showed longer latencies to find the submerged platform in the northwest (NW) quadrant (erroneously called "squadrant" in the paper). During the next stage they tested for cognitive flexibility by moving the platform the northeast (NE) quadrant which required the mice to let go of their learned association and learn a new association. APAP treated mice took longer, on average, to find the new platform, suggesting they suffered from cognitive inflexibility.

Evidence of oxidative stress was evaluated by measuring the *Nrf2/Keap1* transcript ratio using quantitative RT-PCR. The ratio was significantly elevated at 6 hours after the two APAP injections supporting the hypothesis that there is measurable oxidative stress in the brains of young mice exposed to APAP. The authors measured synaptic markers (GLUR1, GAP-43, synaptophysin) using Western blot and found no significant alterations.

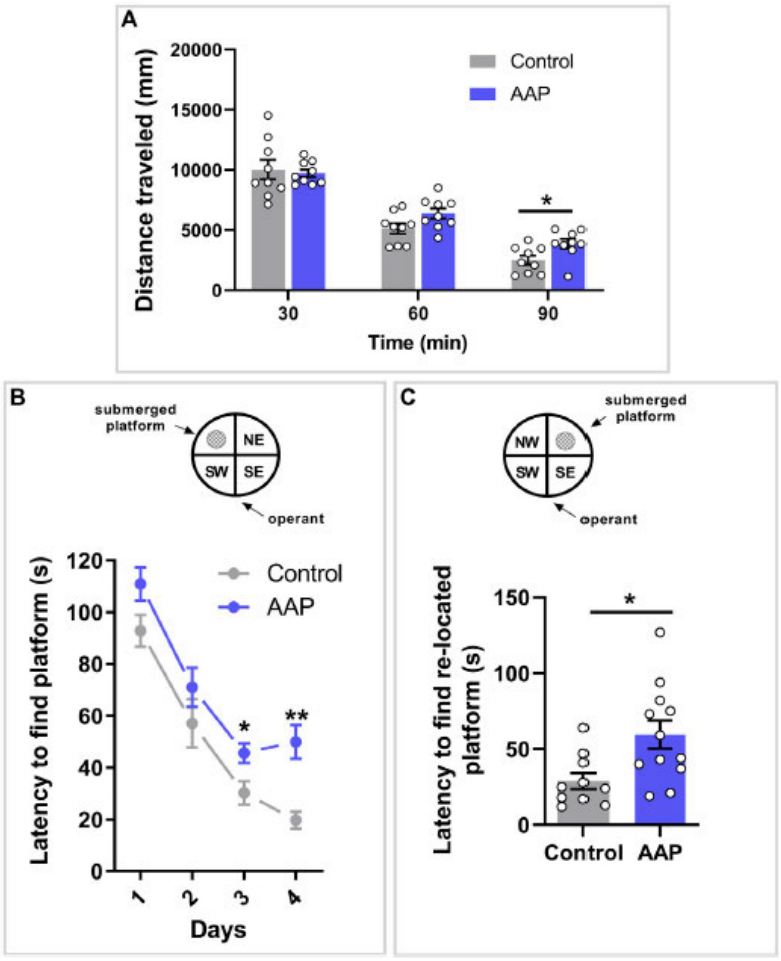


*Figure 38. Behavioral assessments in adulthood showing neonatal exposure to APAP. (A) Spontaneous behavior in 3-month-old mice exposed to vehicle or AAP (30 + 30 mg/kg, 4 h apart) on PND 10. Distance traveled (mm) during each of the three time spells were measured when the mice were introduced to a new home cage (n = 10). (B) Swim maze performance in four-month-old mice (n = 10–12) exposed to vehicle or AAP (30 + 30 mg/kg, 4 h apart) on PND 10. Latencies (s) to locate the platform were measured during the 4 days acquisition period (trials 1–20) and (C) during re-learning period on the fifth day (trials 21–25). Statistical differences are indicated as * if significantly different vs. controls $p \leq 0.05$ and ** if significantly different vs. controls $p \leq 0.01$. Bars represent means ± SEM. Reproduced from Philippot et al., 2022.*

Limitations of this study are that only male mice were tested and route of administration was injection. It is seen as a limitation that the researchers did not perform a probe trial in the Morris Water Maze task as a classic measure of longer-term memory. Otherwise, the implications of this study are that low dose APAP exposure at a timepoint that is relevant to birth in a human from a brain development standpoint (end of 3rd trimester or early postnatal), can cause oxidative stress and disrupting neurocognitive and motor behavior. Strengths are the use of a commons learning and memory paradigm and relatively low dose of APAP. This study was not included in the FDA's review of APAP's neurotoxic effects.

Parameter	Scoring (Philippot et al., 2022)	Score
Direction of Effect	[APAP DNT (+) or not DNT (-)]	+
Controls	[Insufficient (0) Acceptable (1) Good (2)]	2
Sample Size	[Insufficient (0) Acceptable (1) Good (2)]	1.5
Outcomes	[Poor quality (0) Moderate Quality (1) Good Quality (3)]	2
Multi-Dose	[One dose (0) Multi dose (1)]	0
Blinding	[No blinding mentioned or conducted (0) Some Blinding (1) All "Blindable" Parameters Blinded (2)	1
Bias/Conflict Flag	Asterisk, does not alter score	**Total 6.5**

Harshaw & Warner, 2022 *Pharmacology Biochemistry and Behavior*. In Harshaw & Warner, early postnatal mice were randomized to receive one of four treatment conditions; they were given either an injection of a proinflammatory cytokine (0.2 μg/kg dose of IL-1β) or saline vehicle followed by APAP (103.9 mg/kg) or saline vehicle four hours later, on PND 5, 8, and 11 via subcutaneous injection. The four conditions, accordingly, were: I. Vehicle then Vehicle; II. Vehicle then APAP; III. IL-1β then Vehicle, and IV. IL-1β then APAP. Researchers then subjected the mice to behavioral testing for ultrasonic vocalization (PND 7, 9, 12), mini-open field (PND 25-32), social interaction test (PND 27-34), social novelty (PND 45-53), marble burying (PND55-65), automated hole board (PND 60-68), and open field test (PND 65-75). The data were analyzed using a mixed effects linear model after sex-related variables were normalized as Z-scores, and litter and within-cage testing order were considered random effects. Repeated measures and sex interactions were factored in when appropriate.

Researchers noted that APAP treated mice significantly showed more repetitive 360° circling behavior in the marble burying task, but they did not show altered marble burying behavior. APAP treatment was associated with significant elevation of anxiety-related behavior which manifested as enhanced thigmotaxis behavior across arena-based tests such as the open field and hole board tests (staying close to the walls of the arena and avoiding the open center areas). In the social interaction test, APAP exposed mice exhibited an avoidant phenotype characterized of less social engagement while in proximity, and longer latencies to engage when introduced to a novel age/sex/strain matched mouse.

Strengths of this study are the careful and extensive behavioral testing, large sample sizes, and attention to sex differences. The evaluation of the interaction between a pro-inflammatory state and APAP independently and combined is also a strength. These strengths support top scores in controls, sample size, and outcomes. In addition, the authors [describe blinding] in support of a score of 2 for blinding.

Some limitations of this study are that many of the variables included (which were numerous) were complex that they distilled them via factor analysis making the dissemination of their results to broader audiences challenging. Controlled preclinical mouse studies should ideally be immune to the need for latent variable approaches and over-cumbersome statistical approaches if for no other reason than they further distance the literature from audiences that can use them. I was not able to find ultrasonic vocalization data from the APAP treatment vs control; perhaps it

was not performed. These limitations do not justify lowering the scores as their strengths outweigh these issues.

Finally, authors state "A final issue is that most rodent studies, to date, have failed to examine early life APAP in the biologically relevant context in which most exposure occurs in humans—i.e., fever and/or inflammation." This is one take, but one that is a bit narrow. Pregnant people take APAP for various reasons – headaches, muscle, joint aches, etc. So, while I agree that looking at the interactive effects of immune activation/inflammation or other background stressors and developmental APAP exposure it is not necessary for preclinical models under many circumstances. These are not score-driving comments, rather points of clarification. This study was not included in the FDA's review of APAP's neurotoxic effects.

Parameter	**Scoring (Harshaw & Warner, 2022)**	**Score**
Direction of Effect	[APAP DNT (+) or not DNT (-)]	+
Controls	[Insufficient (0) Acceptable (1) Good (2)]	2
Sample Size	[Insufficient (0) Acceptable (1) Good (2)]	2
Outcomes	[Poor quality (0) Moderate Quality (1) Good Quality (3)]	3
Multi-Dose	[One dose (0) Multi dose (1)]	0
Blinding	[No blinding mentioned or conducted (0) Some Blinding (1) All "Blindable" Parameters Blinded (2)	2
Bias/Conflict Flag	Asterisk, does not alter score	**Total 9**

Baker et al., 2023 *Neurobiology of Disease*. In 2023, my research group published a study where we evaluated the influences of a single 150 mg/kg APAP dose administered via a flavored gelatin chewable to pregnant mice. We found sex-specific differences in ultrasonic vocalizations, as well as changes in ambulation and rearing in mice treated with APAP. In terms of study design, we chose the dose of 150 mg/kg because that dose has been consistently and appropriately used in the preclinical literature as a non-hepatotoxic dose. We opted to be at the high end of dosing to see an effect if it existed, in line with the principles of precaution and because we were not funded or staffed to perform multiple doses. We initially purchased "timed pregnant" mice from the commercial vendor and began dosing early in pregnancy (embryonic day 4, or E4), but only 4 of those dams were pregnant which created small treatment groups when the dam is an experimental unit. Those dams were randomized to APAP or vehicle control. Accordingly, we bolstered the sample size by generating another cohort with more timed pregnant dams that we likewise randomized to APAP or control groups. However, to ensure that a higher proportion of the dams would be pregnant after shipping, we ordered them to arrive at E7 and began dosing at E10. We evaluated the offspring in a set of various behavioral tasks designed in conjunction with the director of the Columbia Mouse NeuroBehavior Core to capture ADHD-like phenotypes (activity changes, learning, memory, and attention changes) and general neurodevelopmental behaviors. Finally, we performed gene expression profiling (aka transcriptomics) using RNA-sequencing (RNA-seq for short) in the forebrain of adult offspring of the treatment conditions to characterize the longstanding effects of the developmental APAP exposure. As stated previously, we found sex-specific differences in ultrasonic vocalizations, as well as changes in ambulation and rearing

in mice treated with APAP. We also found sex-specific differences in gene expression in mice that received APAP compared to control groups.

Rather than relying on a limited set of toxicological endpoints and risk missing neurodevelopmental phenomena, we opted to perform a more complex analysis. We used transcriptional profiling using RNA-sequencing from the forebrains of adult mice treated with vehicle or APAP prenatally and in early life. RNA-sequencing is a powerful tool in molecular biology and genomics that allows us a comprehensive and unbiased analysis of gene expression patterns.

We analyzed gene expression from the brains of three male and three females from the two conditions and found that the APAP exposure caused lasting gene expression effects across hundreds of genes. Accordingly, it is helpful to look at the classifications of gene groups rather than functions of individual genes. In doing so, we found that some of these expression effects were independent of sex (First column in the figure below [Figure 7 of Baker et al., 2023] with pathways such as "Glutathione metabolism" and "Chemical carcinogenesis" enriched for All sexes) and some that were restricted or more pronounced in one sex or the other. For instance, whereas across both sexes and within females most pathways were upregulated and indicated stress, metabolism, damage, and other chemical responses, in males, in contrast, there were numerous pathways significantly downregulated such as "Steroid biosynthesis" (might, for instance, comport with the literature on androgen effects with APAP), and "Oxidative phosphorylation."

Strengths of this study were the voluntary (non-stressful) oral APAP administration, RNA-seq analyses, ultrasonic vocalizations, measurement of prepulse inhibition, measure of learning, memory, and attention in the 5-choice task. Limitations include a single dose tested and limited sample size for the 5-choice task (due to the months it takes to train and test and the limited number of chambers that were available to the researchers). This study was not included in the FDA's review of APAP's neurotoxic effects.

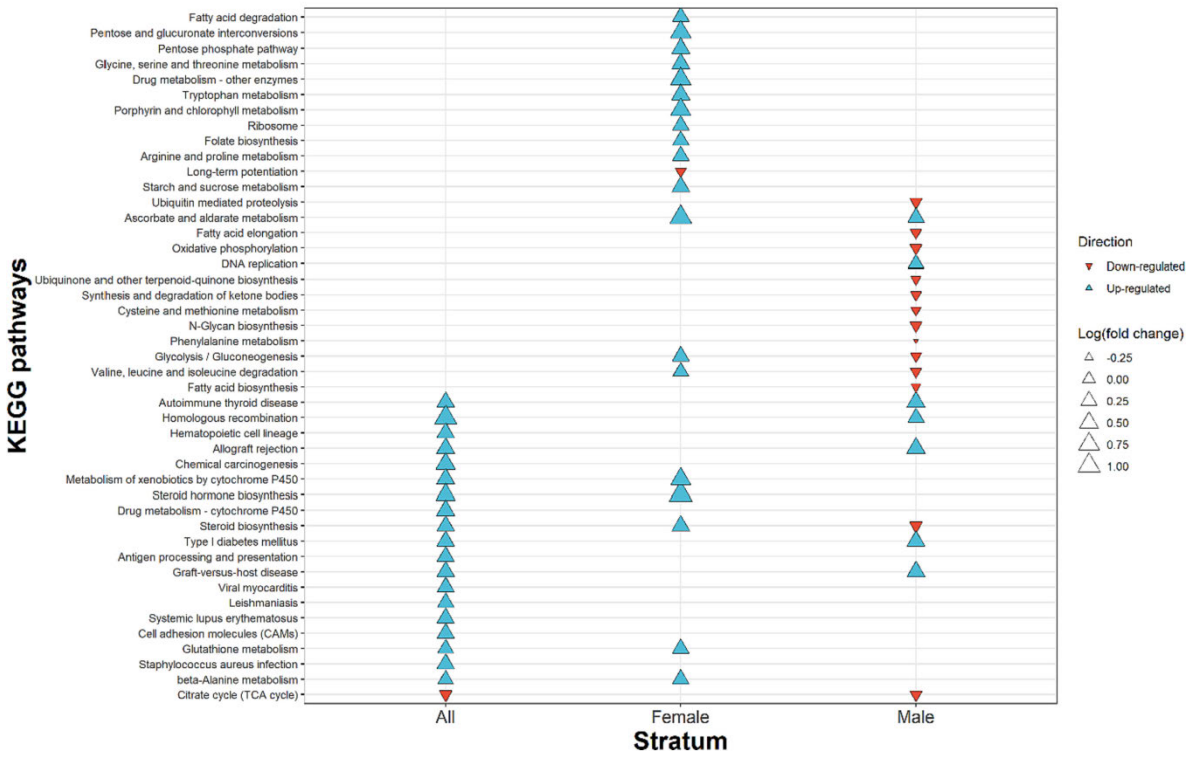
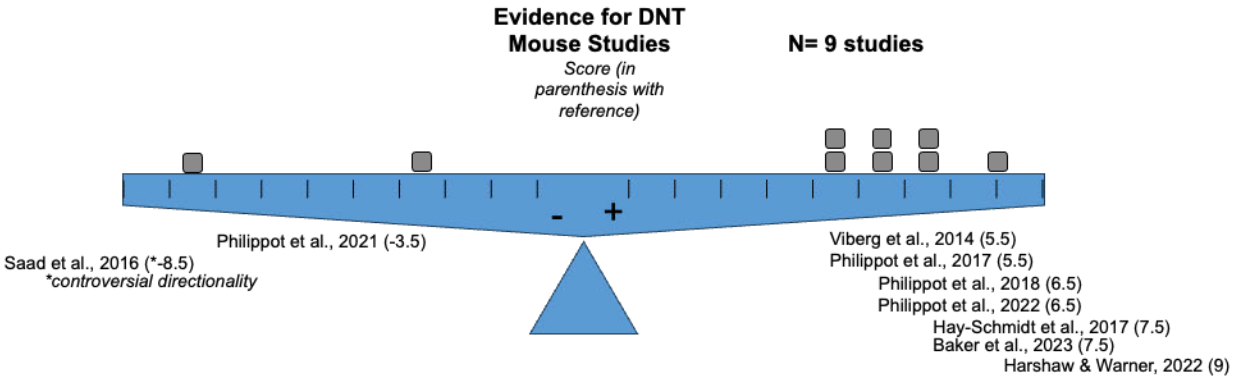


Figure 39. Ensem Ensemble of Gene Set Enrichment Analysis (EGSEA). APAP treatment over control fold changes in top 20 enriched terms for KEGG pathways sorted from top to bottom by EGSEA median ranking score. Analysis repeated for all mice and stratified by sex (n = 24). Reproduced from Baker et al., 2023.

Parameter	**Scoring (Baker et al., 2023)**	**Score**
Direction of Effect	[APAP DNT (+) or not DNT (-)]	+
Controls	[Insufficient (0) Acceptable (1) Good (2)]	2
Sample Size	[Insufficient (0) Acceptable (1) Good (2)]	1.5
Outcomes	[Poor quality (0) Moderate Quality (1) Good Quality (3)]	3
Multi-Dose	[One dose (0) Multi dose (1)]	0
Blinding	[No blinding mentioned or conducted (0) Some Blinding (1) All "Blindable" Parameters Blinded (2)	1
Bias/Conflict Flag	Asterisk, does not alter score	**Total 7.5**

[redacted]



Line of Evidence	Reliability	Relevance	Weight Assigned
In vivo	MEDIUM	HIGH	MODERATE to HIGH

Reliability of the in vivo (mouse and rat) studies is MEDIUM. The potential to generate abundant data with clear reporting exists but not all studies exhibit that characteristic. The RELEVANCE is HIGH as in vivo rodent models are appropriate and outcome variables focus on mechanisms of injury and insult relevant to APAP. Accordingly, the weight assigned for all in vivo lines of evidence is **MODERATE to HIGH**

B. IN VITRO/EX UTERO/EX VIVO STUDIES

Stark et al., 1989 *Toxicol Appl Pharmacol* [Stark et al., 1989a]. Stark and colleagues collected embryonic day 9 embryos (also called "conceptus") from timed pregnant rats (female rats that were pregnant at a known stage because the point of conception was known) that were then cultured in cell culture conditions for further chemical dosing and evaluation of development and morphology. The authors were comparing APAP and its de-acetylated metabolite PAP to a known teratogen 7-hydroxy-2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (7-OH-AAF or simply "AAF") as both they had observed similar impacts on neural tube ductal closures in cultured rat embryos with exposure to both AAF and APAP. In the current study, the researchers measured embryo length, proportion of cultured embryos that were viable (alive) per treatment condition, and the proportion that showed developmental malformations – those that either did not exhibit appropriate and complete axial rotation (Figure inset below provided for illustrative purposes), or those that had open anterior neuropores (meaning that the neural tube failed to close on the anterior end), upon exposure to the test substances at various concentrations (APAP tested at 0.1, 0.5, and 1.0 mM), at embryonic day 10.

Their results showed that APAP affected embryo length at 0.1 mM and above and affected morphological variables at doses above 0.1 mM. PAP, on the other hand, caused axial rotation and neural tube abnormalities at much lower doses- at 0.01, and 0.05 mM, or 10 µM and 50 µM respectively. PAP is generated in vivo from the deacetylation of APAP by hydrolysis. It is not clear if PAP is generated at notable levels in embryos based on the enzymatic and biochemical studies performed by Stark and colleagues. Nevertheless, their experiment shows that APAP and

its deacetylated metabolite are capable of altering embryonic growth and development and could act as a neural tube teratogen.

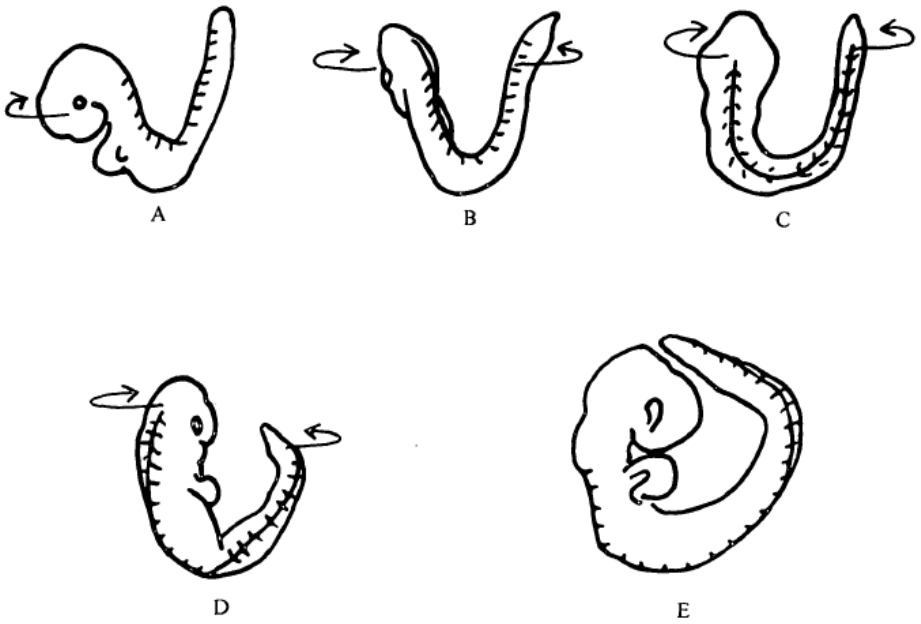


Fig. 2. Diagrams to show the stages of rotation of the embryonic axis that are referred to in Table 1. Position of mid-dorsal surface is indicated by hatched line. A, Not turned; B, ¼ turned; C, ½ turned; D, ¾ turned; E, fully turned.

Figure 40. Modified from Deucher 1975.

Parameter	**Scoring (Stark et al., 1989a)**	**Score**
Direction of Effect	[APAP DNT (+) or not DNT (-)]	+
Controls	[Insufficient (0) Acceptable (1) Good (2)]	2
Sample Size	[Insufficient (0) Acceptable (1) Good (2)]	1
Outcomes	[Poor quality (0) Moderate Quality (1) Good Quality (3)]	3
Multi-Dose	[One dose (0) Multi dose (1)]	1
Blinding	[No blinding mentioned or conducted (0) Some Blinding (1) All "Blindable" Parameters Blinded (2)	2
Bias/Conflict Flag	Asterisk, does not alter score	**Total 9**

Stark et al. 1989 *Biochemical Pharmacology* [Stark et al., 1989b]. In a study published in Biological Pharmacology (Volume 38(16): 2685-2692) soon after the Stark et al., 1989a study described above, using a similar design, rat E10 embryos were exposed to APAP and its effect on embryo morphology and neurulation was evaluated much the same as the prior study methodologically. However, in this study, they were interested in understanding how depletion of reduced GSH would affect APAP's embryotoxicity (as a function of APAP bioactivation and intermediates targets and detoxification). They evaluated pre-treatment with L-buthionine-S,R-sulfoximine (BSO), a compound which inhibits one of the enzymes that synthesizes GSH.

APAP was radiolabeled. Radiolabeling is a term to describe labeling with a radioactive isotope, in this case) ([3H]APAP). This process allowed the authors to trace the distribution of APAP and APAP metabolites within the embryos. The embryos were then fractionated using a gradient separation technique, allowing for separation of different cellular components based on their density. This process was employed to determine gradient fractionated which molecules APAP metabolites/intermediates covalently bind and complex with. They found that radioactivity was restricted to protein-rich embryo homogenate fractions, showing that the reactive electrophile intermediates of APAP were bound to protein. This finding is key because APAP's covalent binding to proteins could lead to protein damage, disruption of cellular functions, and activation of cellular stress.

Pretreatment with BSO to deplete GSH markedly increased this radioactivity demonstrating that the amount of NAPQI adduct formation to protein was higher in the presence of lower GSH. In other words, APAP was more likely to bind to proteins within these cells when GSH was lower, further reinforcing evidence that depleted GSH leaves cells vulnerable to APAP damage. Pretreatment with BSO (and GSH reduction) also enhanced APAP's toxic effects in embryological parameters such as reduced protein content, embryo length, and abnormal open neural tubes (see Figure 5 from the original paper).

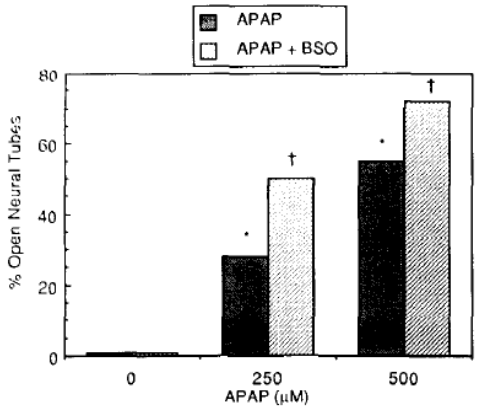


Fig. 5. Incidence of abnormally open anterior neural tubes following 24-hr exposure to APAP alone or in combination with GSH depletion. BSO denotes pre-exposure to the conceptus to 0.5 mM BSO for 16 hr prior to addition of APAP. Key: (*) significantly ($P \leq 0.05$) different from control; and (†) significantly ($P \leq 0.05$) different from APAP exposure alone. Pretreatment with BSO alone had no significant effect ($P > 0.05$) on the incidence of abnormal neurulation.

Figure 41. Reproduced from Stark et al., 1989b.

Parameter	Scoring (Stark et al., 1989b)	Score
Direction of Effect	[APAP DNT (+) or not DNT (-)]	+
Controls	[Insufficient (0) Acceptable (1) Good (2)]	2
Sample Size	[Insufficient (0) Acceptable (1) Good (2)]	1
Outcomes	[Poor quality (0) Moderate Quality (1) Good Quality (3)]	3
Multi-Dose	[One dose (0) Multi dose (1)]	1
Blinding	[No blinding mentioned or conducted (0) Some Blinding (1) All "Blindable" Parameters Blinded (2)	2
Bias/Conflict Flag	Asterisk, does not alter score	**Total 9**

Buzanska et al., 2009 *Stem Cells*. Buzanska and colleagues used human umbilical cord blood-derived neuronal stem cells to derive neuronal stem cells (HUCB-NSC) as a model for developmental neurotoxicity (DNT). HUCB-NSCs are a helpful research tool for neurotoxicity because they can differentiate into many kinds of neural cells, including neurons and glial cells, allowing visibility into the way neurotoxins can affect various cell systems. In this study, several various chemicals were tested in their NSC model including APAP which was included as a negative control. APAP doses tested were 1-1000 µM. Only the highest dose of APAP altered any of the parameters tested – specifically, cell viability under any of the NSC differentiation conditions. For instance, if the NSCs were given a cocktail of factors to direct them to a neuronal fate or to glial (non-neuronal, such as oligodendrocyte or astrocyte) fate, only the 1000 µM dose was statistically cytotoxic. Since that dose of APAP is substantially higher than that which would be achieved in a therapeutic clinical dose of APAP, then this experimental system supports that APAP is not neurotoxic to these cells under these conditions. Some limitations of this study include that hepatic or placental metabolism or biotransormation are missing, so the toxicity of APAP metabolites has not been evaluated.

Parameter	Scoring (Buzanska et al., 2009)	Score
Direction of Effect	[APAP DNT (+) or not DNT (-)]	-
Controls	[Insufficient (0) Acceptable (1) Good (2)]	2
Sample Size	[Insufficient (0) Acceptable (1) Good (2)]	2
Outcomes	[Poor quality (0) Moderate Quality (1) Good Quality (3)]	1.5
Multi-Dose	[One dose (0) Multi dose (1)]	1
Blinding	[No blinding mentioned or conducted (0) Some Blinding (1) All "Blindable" Parameters Blinded (2)	0
Bias/Conflict Flag	Asterisk, does not alter score	**Total –6.5**

Posadas et al., 2010 *PLoS ONE*. (In vitro and in vivo testing was performed in this study, see independent entries for this study). In their in vitro experiments, Posadas and colleagues used cultured rat cortical neurons to evaluate the neurotoxicity of APAP in vitro. They grew mixed cortical cultures that were enriched in neurons (95%) and waiting to 7-10 days in vitro (DIV) in order to let the cells mature at which time they dosed them with vehicle (1% dimethylsufloxide, aka DMSO) as a control or 0.5-10 mM APAP and tested cell viability using a lactate

dehydrogenase (LDH) assay, a classic measure of cell viability. Relative LDH release indicates cellular damage as it is normally an intracellular enzyme that is maintained solely within cells that are intact and healthy. Upon cell death and membrane damage, the extracellular levels of LDH increase. Posadas and colleagues that a single 1 mM dose of APAP causes an increasing level of LDH release over a timescale of 6-24 hours after dosing with statistically significant elevations noted at 18 and 24 hours. Upon testing a range of APAP doses at a single time point of 24 hours, they noted a significant increase in LDH release at all concentrations tested (0.5-10 mM). They next looked for apoptosis (programmed cell death) through by running purified DNA from cellular homogenates in a gel electrophoresis preparation and visualizing the DNA pattern and they saw "laddering" of the DNA that is consistent with apoptosis at doses of 0.5 mM and above. Additionally, by growing cortical cells on glass coverslips and labeling the DNA with Hoechst 33342 – a DNA dye, and visualizing on a fluorescence microscope, they noted that APAP causes DNA fragmentation consistent with apoptosis at doses of 0.5 mM and above. Finally, Posadas et al., evaluated the involvement of the mitochondria and oxidative stress in the toxicity of APAP in the rat cortical neurons by measuring cytochrome c, caspase 3, and the fluorescent dye CM-H2-DCFDA - markers of mitochondrial damage, mitochondrial driven apoptosis, and reactive oxygen species, respectively. All were elevated by APAP. They also showed that Cyp2e1 is expressed in these embryonic rat cortical cells at baseline and APAP elevated its expression indicating that toxicity could be driven by local metabolism to NAPQI in the brain and/or that GSH depletion could underlie neurotoxicity. One limitation of this study was much of the details of the methods was referred to by citing previous studies, which in some cases, also referred back to previous studies (e.g., the primary cortical cell culture methods).

Parameter	**Scoring (Posadas et al., 2010) [In vitro component]**	**Score**
Direction of Effect	[APAP DNT (+) or not DNT (-)]	+
Controls	[Insufficient (0) Acceptable (1) Good (2)]	1
Sample Size	[Insufficient (0) Acceptable (1) Good (2)]	2
Outcomes	[Poor quality (0) Moderate Quality (1) Good Quality (3)]	3
Multi-Dose	[One dose (0) Multi dose (1)]	1
Blinding	[No blinding mentioned or conducted (0) Some Blinding (1) All "Blindable" Parameters Blinded (2)	0
Bias/Conflict Flag	Asterisk, does not alter score	**Total 7**

Schultz et al., 2011 *Basic & Clinical Pharmacology & Toxicology*. In a small study to characterize whether APAP, its deacetylated PAP metabolite, or anandamide were toxic to neurons, Schultz and colleagues prepared mouse primary embryonic cortical cultures in multiwell cell culture plates and administered these substances at the third "day in vitro" (DIV3). They then performed a simple live/dead assay with two fluorescent dyes – one that labels dead cells, and one that labels all cells, and then imaged the cells on a microscope to count the proportion of cells that were dead. They found that APAP was not toxic at any dose tested (range 1-100 µg/ml [or ~ 6.6 - 662 µM], but PAP caused significant cell death starting at 1 µg/ml.

The take home message here is that APAP as a parent molecule was not toxic at the doses and conditions tested but the deacetylated metabolite (p-aminophenol/PAP) appears to be

neurotoxic to developing neurons in vitro. Note, this culture system contained approximately 93% neurons but was otherwise a mixed cortical culture system.

Some limitations of this publication are that they do not give replication error estimates for the toxicity measures for APAP and PAP. Testing for neurotoxicity at DIV3 is potentially too early or at least might need to be interpreted carefully as primary cortical cultures do not mature until at least DIV7 at which point they are beginning to establish connections and synapses and establish burst activity/coordinated circuits. Details on what constituted the vehicle control are missing as are other details such as replicate numbers and sample sizes.

Parameter	**Scoring (Schultz et al., 2011)**	**Score**
Direction of Effect	[APAP DNT (+) or not DNT (-)]	-
Controls	[Insufficient (0) Acceptable (1) Good (2)]	1
Sample Size	[Insufficient (0) Acceptable (1) Good (2)]	0.5
Outcomes	[Poor quality (0) Moderate Quality (1) Good Quality (3)]	1
Multi-Dose	[One dose (0) Multi dose (1)]	1
Blinding	[No blinding mentioned or conducted (0) Some Blinding (1) All "Blindable" Parameters Blinded (2)	0
Bias/Conflict Flag	Asterisk, does not alter score	**Total -3.5**

Wu et al., 2016 *BMC Pharmacology and Toxicology*. Wu and colleagues used commercial human stem cell derived cultured neurons to screen neurotoxic potential of various chemicals and used APAP as a putative non-neurotoxic control. The obtained measures of neuronal cell morphology using high-content imaging which is a high-resolution microscopy technique. The tested concentrations ranging from 1 to 10 µM. APAP at those concentrations did not affect any measures of cell count or density, or properties of the neurites such as neurite length, neurites per neuron, or branchpoints per neuron. Authors did see alterations to these parameters with other compounds tested such as endocrine disruptors including estradiol and bisphenol A at similar concentrations, demonstrating that they are capable of measuring changes in these variables. Strengths of this study are the high content imaging tool. Weaknesses are sample sizes were only two biological replicates, no blinding, and very small doses of APAP that are too low to reflect a full range of plasma and CSF levels and thus limiting conclusions on APAP neurotoxicity.

Parameter	Scoring (Wu et al., 2011)	Score
Direction of Effect	[APAP DNT (+) or not DNT (-)]	-
Controls	[Insufficient (0) Acceptable (1) Good (2)]	2
Sample Size	[Insufficient (0) Acceptable (1) Good (2)]	1
Outcomes	[Poor quality (0) Moderate Quality (1) Good Quality (3)]	1
Multi-Dose	[One dose (0) Multi dose (1)]	1
Blinding	[No blinding mentioned or conducted (0) Some Blinding (1) All "Blindable" Parameters Blinded (2)	0
Bias/Conflict Flag	Asterisk, does not alter score	**Total -5**

Labba et al., 2022 *Toxicology and Applied Pharmacology*. Labba and colleagues utilized two model systems of neural cells to test the hypothesis that APAP's mechanism of neurotoxicity is via disturbances to neurite (neuronal processes such as axons and dendrites). They posit that APAP could be exacting its toxic effects via actions on the actin binding protein SPTBN1, which is implicated in neurodevelopmental disorders and, as reviewed in their paper, has been shown to be altered in peripheral cells. They cultured primary chicken granule cell neurons as well as human NTERA2 cells (NT2Ns) which are an immortalized human embryonic carcinoma cell line that can be differentiated into human neuron-like cells that gain neuronal morphology and express neuronal gene expression patterns. Equipped with these two cell models, they dosed APAP at concentrations ranging from 100 to 1600 µM for 24 to 72 hours. They justified these doses as representing therapeutic (100-200 µM) and overdose (≥800 µM) concentrations. Following these intervals, they evaluated cell viability with the MTT assay and DNA content, cell morphology (neurite lengths, and branch points) with live cell imaging using a sophisticated and modern imaging system, cell migration from spheroids, and protein abundances of SPTBN1 and TUBB3 which labels beta-tubulin. By performing fluorescence immunolabeling they can visualize the abundance, distribution and quality of the cytoskeletal proteins in these neuronal (or neuronal-like) cells as a function of APAP treatment.

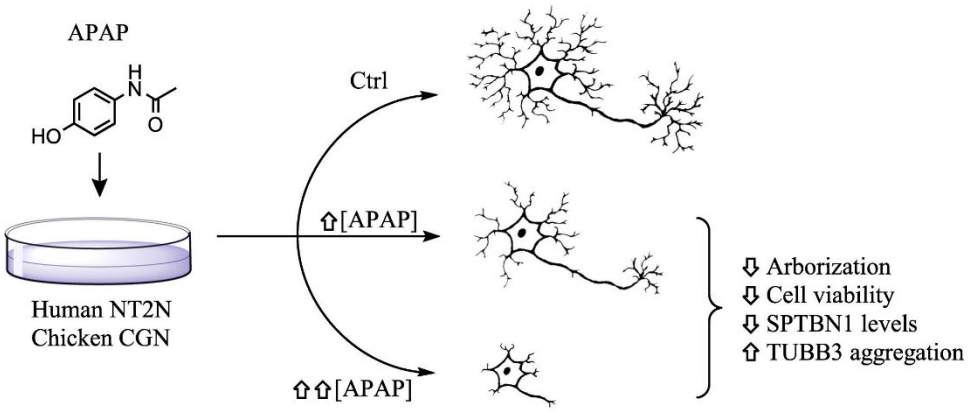


Figure 42. Visual abstract.
Reproduced from Labba et al., 2022.

APAP caused cell model-specific viability differences that were time-dependent. When relying on the MTT assay readout, at the earliest timepoints, APAP did not cause appreciable cell death at any concentration in either cell model. At 48 hours, APAP was toxic at higher doses only in the NT2N model but not the CGN cells. Finally, APAP showed concentration dependent toxic effects in both cell models after 72 hours of administration. In contrast, the DNA content measure showed effects at earlier timepoints. APAP had significant effects on neurite lengths and branching, disturbed cell migration, and reduced the abundance of SPTBN1 and increase the abundance of TUBB3 positive puncta. This study provides evidence that APAP can inhibit the growth of neuronal processes and branching potentially through critical actin and tubulin proteins. This has implications for intracellular trafficking and cellular structure. The study also demonstrated that cell migration was inhibited by APAP which also has major implications for neurodevelopmental disorders such as ASD and ADHD for which cellular migration deficits have been implicated.

Strengths of this study are that they contrasted toxicity effects such as neurite influences relative to viability influences in two separate in vitro model systems. Their immunolabeling is excellent and they provide useful control photomicrographs in the supplement. They also used very conservative statistics looking at interactions of time and treatment levels.

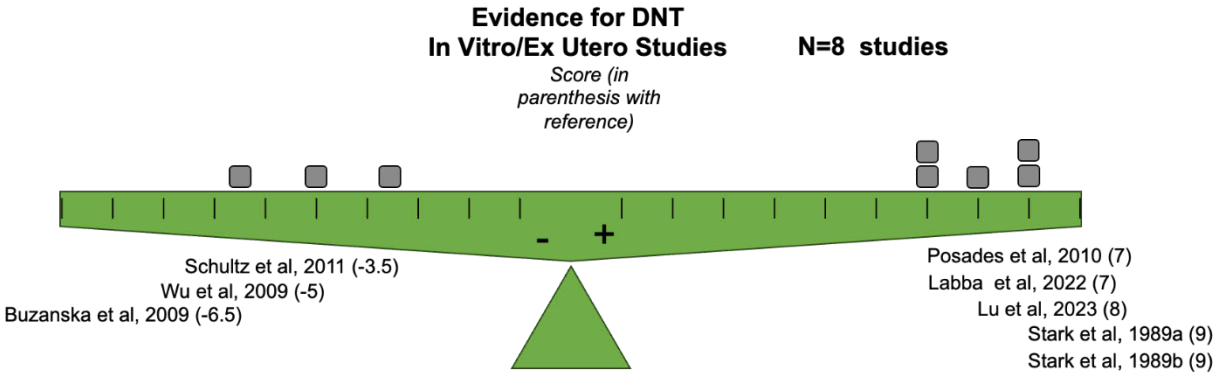
Weaknesses of the study is that there appears to have been no blinding of the experimenters. The cellular models themselves are limited in the fact that one is chicken derived and the other is human cancer derived and not of CNS origin which can limit the relevance of the findings.

Parameter	**Scoring (Labba et al., 2022)**	**Score**
Direction of Effect	[APAP DNT (+) or not DNT (-)]	+
Controls	[Insufficient (0) Acceptable (1) Good (2)]	2
Sample Size	[Insufficient (0) Acceptable (1) Good (2)]	1
Outcomes	[Poor quality (0) Moderate Quality (1) Good Quality (3)]	3
Multi-Dose	[One dose (0) Multi dose (1)]	1
Blinding	[No blinding mentioned or conducted (0) Some Blinding (1) All "Blindable" Parameters Blinded (2)	0
Bias/Conflict Flag	Asterisk, does not alter score	**Total 7**

Lu et al., 2023 *Cells*. Lu et al used human iPSC-derived neurons and astrocytes co-cultures in 2D and 3D format to test various chemicals for electrophysiological alterations, using APAP and aspirin (up to 100 µM) as negative controls as they consider these non-neurotoxic substances. The depolarization activity of the cultured cells and spheroids was evaluated by loading calcium sensitive dyes and performing time lapse fluorescence microscopy and measuring the calcium transient intensities. When neurons depolarize during action potentials, calcium influxes into the cells. When calcium sensitive dyes are introduced into the cells, they fluorescence at higher intensities during burst activity, action potentials, and any cellular depolarization. They found that the highest dose tested of 30 µM significantly decreased peak number (the number of calcium bursts) in 2D cultures (average of 94.5% decrease) and significantly increased (average of 47.2% increase) in the peak number of calcium bursts in 3D cultures. Supplementary data show that APAP caused significant decreases in peak metrics including Area Under the Curve (AUC), Peak

to Peak Interval, Peak Width, and Peak Amplitude only for 2D cultures. The authors of that study, Janssen employees, argued that the neuro-active properties of APAP in vitro must be due to the "very high concentration" used in their control model. However, 30 µM is well within the concentration seen at therapeutic doses in human patients (showing Pediatric CSF APAP levels ranged from 1.3 and 18 mg/L which is 8.6 - 119 µM) (Kumpulainen et al., 2007). This study reflects that even in an in vitro model and at therapeutic doses, APAP is causing neurophysiological changes. Accordingly, the notion that APAP is a benign, non-neurotoxic, non-neuroactive chemical in nervous system tissues is unsupported. Strengths of this study are the use of functional neurophysiological measures in human stem cell derived neuron astrocyte co-cultures and blinded chemical libraries for dosing at an outside contract research organization. Weaknesses are a single cell line origin from one sex and what appears to be critical missing information on sample size.

Parameter	**Scoring (Labba et al., 2022)**	**Score**
Direction of Effect	[APAP DNT (+) or not DNT (-)]	+
Controls	[Insufficient (0) Acceptable (1) Good (2)]	2
Sample Size	[Insufficient (0) Acceptable (1) Good (2)]	0
Outcomes	[Poor quality (0) Moderate Quality (1) Good Quality (3)]	3
Multi-Dose	[One dose (0) Multi dose (1)]	1
Blinding	[No blinding mentioned or conducted (0) Some Blinding (1) All "Blindable" Parameters Blinded (2)	2
Bias/Conflict Flag	Asterisk, does not alter score	**Total 8**


Evidence for DNT
In Vitro/Ex Utero Studies
N=8 studies
Score (in parenthesis with reference)
-
+
Schultz et al, 2011 (-3.5)
Wu et al, 2009 (-5)
Buzanska et al, 2009 (-6.5)
Posades et al, 2010 (7)
Labba et al, 2022 (7)
Lu et al, 2023 (8)
Stark et al, 1989a (9)
Stark et al, 1989b (9)

Line of Evidence	**Reliability**	**Relevance**	**Weight Assigned**
In Vitro/Ex Utero	HIGH	MEDIUM	MODERATE

Reliability is HIGH as in vitro and ex utero studies are performed with excellent level of control and standards. Relevance is MEDIUM as cellular, molecular, and embryonic effects can be readily measured with precise control overdosing conditions, the environment has limitations in full multi-system organismal biology and is performed in in vitro environments that so not perfectly recapitulate in utero conditions. Therefore, overall weight assigned is **MODERATE.**

C. IN SILICO DATA

ToxCast. The U.S. Environmental Protection Agency along with other regulators (including the FDA) have leveraged substantial expertise and resources in predictive toxicology tools and databases that can aid in filling data gaps about the toxicity and safety of pharmaceuticals and environmental chemicals. The Toxicity Forecasting program (ToxCast) (https://comptox.epa.gov/dashboard/) has combined high throughput libraries of chemicals for screening of environmental chemicals including agrochemicals, orphan drugs, industrial chemicals, food additives, and various other agents such as dyes to create rich datasets to perform predictive toxicology using read-across tools (Williams et al., 2017).

The ToxCast dashboard shows that APAP has potent activity for androgen receptor (AR), nuclear receptor family, in general (inclusive of many hormone receptors including sex steroids and stress hormones), cytochrome P450 enzymes, and finally SOX1 activity which is a developmentally important transcription factor implicated in neurodevelopmental disturbances, respectively (see the table below. Note, APAP has been tested in 979 assays, the four below are the ones in which APAP showed significant "Active" calls. AC50 refers to the concentration at 50% maximum activity, or a measure of relative potency, in micromolar (µM) units).

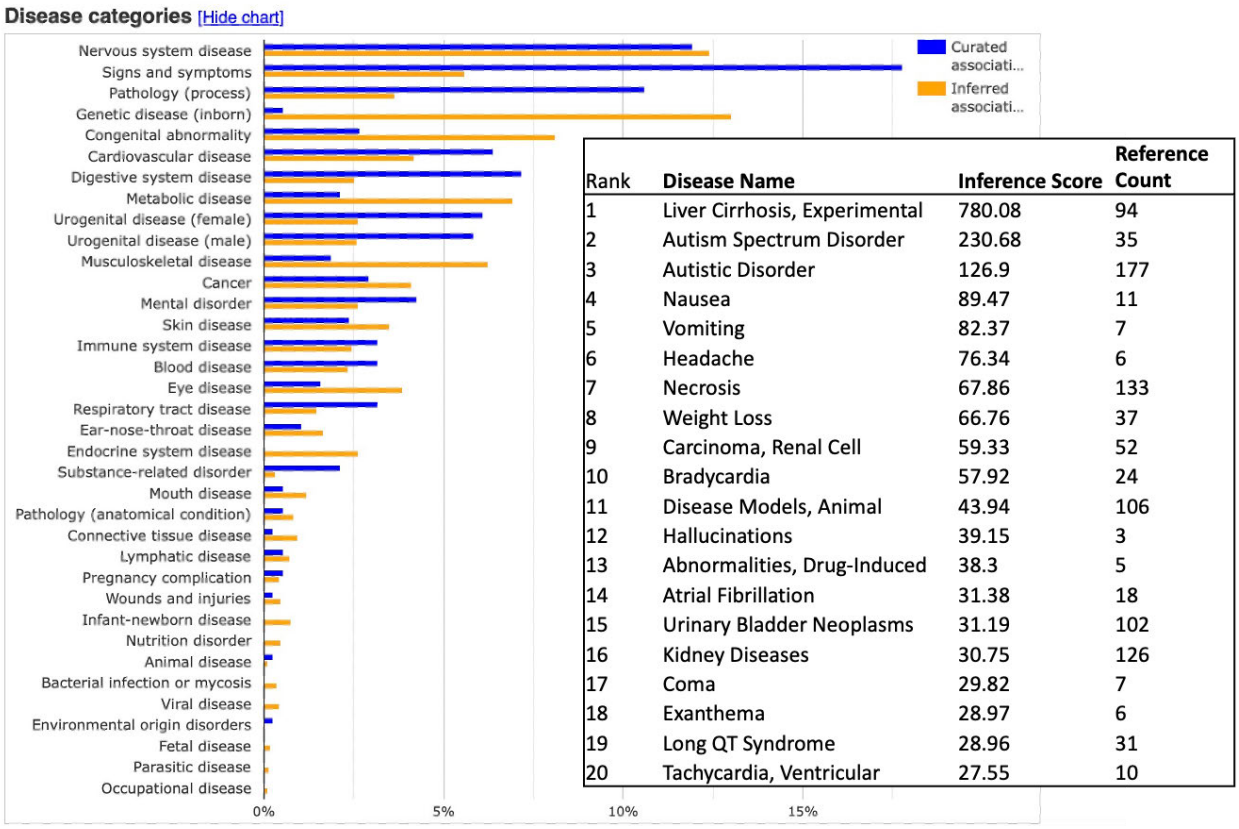
NAME	Description	AC50	INTENDED TARGET FAMILY
UPITT_HCI_U2OS_AR_TIF2_Nucleoli_Antagonist	Data from the assay component UPITT_HCI_U2OS_AR_TIF2_Nucleoli_Antagonist was analyzed into 1 assay endpoint. This assay endpoint, UPITT_HCI_U2OS_AR_TIF2_Nucleoli_Antagonist, was analyzed in the positive fitting direction relative to DHT as the baseline of activity. Using a type of binding reporter, measures of receptor for loss-of-signal activity can be used to understand the binding at the pathway-level as it relates to the gene AR.	0.25116021	nuclear receptor
NVS_NR_hPR	Data from the assay component NVS_NR_hPR was analyzed into 1 assay endpoint. This assay endpoint, NVS_NR_hPR, was analyzed in the positive fitting direction relative to DMSO as the negative control and baseline of activity. Using a type of binding reporter, loss-of-signal activity can be used to understand changes in the binding as they relate to the gene PGR. Furthermore, this assay endpoint can be referred to as a primary readout, because the performed assay has only produced 1 assay endpoint. To generalize the intended target to other relatable targets, this assay endpoint is annotated to the "nuclear receptor" intended target family, where the subfamily is "steroidal".	14.3629481	nuclear receptor
LTEA_HepaRG_CYP1A1_up	Change in transcription factor expression relative to control (delta-delta-ct) for HepaRG cell cultures in an induction preparation. The adherent cells have some metabolic capability. Expression measured by inducible reporter assay using Fluidigm qRT-PCR to monitor. Suffix _up indicates curve fitting for increase in expression (induction).	34.0111873	cyp

ATG_Sox_CIS_up	Data from the assay component ATG_Sox_CIS was analyzed into 1 assay endpoint. This assay endpoint, ATG_Sox_CIS_up, was analyzed in the positive fitting direction relative to DMSO as the negative control and baseline of activity. Using a type of inducible reporter, measures of mRNA for gain-of-signal activity can be used to understand the reporter gene at the transcription factor-level as they relate to the gene SOX1. Furthermore, this assay endpoint can be referred to as a primary readout, because this assay has produced multiple assay endpoints where this one serves a reporter gene function. To generalize the intended target to other relatable targets, this assay endpoint is annotated to the dna binding intended target family, where the subfamily is HMG box protein.	85.3541036	dna binding

The Comparative Toxicogenomics Database. Integration of data across studies and data resources is challenging. Researchers at North Carolina State University, with funding and support of the NIH, generated the Toxicogenomics Database (CTDbase.org) to integrate knowledge across domains about genetics, exposures, model systems, and other lines of evidence that renders a powerful tool for evidence mapping, systems biology, discovery, disease relevance and literature discovery. The CTDbase is a publicly available, government-funded resource that collects and integrates information from scientific literature to provide insights into the relationships between chemicals, genes, diseases, and other biological entities. It serves as a valuable tool for researchers, scientists, and anyone interested in understanding the molecular mechanisms underlying toxicological and environmental health effects.

CTDbase can be searched by chemical (or gene, or disease, and so forth) and fully orthogonal types of data linking to the other domains are linked and clickable from one's search. When searching CTDbase for disease links for APAP, the results show, based on gene pathway involvement, expected hepatic targets but also strong neurodevelopment (autism, ASD) enrichment.

In the results for APAP, below disease categories on left show nervous system disease as a top if not the top category. Results of the top 20 tabular specific enrichments are shown in the inset to the right. Rank 2 and 3 are autism or ASD. These connections show enriched inference scores that are based on strength of network enrichments vs a background of random networks. Put more plainly, the inference score refers to a scoring system used to evaluate the strength of evidence supporting the association between a specific chemical and a particular biological event or outcome.



Rank	Disease Name	Inference Score	Reference Count
1	Liver Cirrhosis, Experimental	780.08	94
2	Autism Spectrum Disorder	230.68	35
3	Autistic Disorder	126.9	177
4	Nausea	89.47	11
5	Vomiting	82.37	7
6	Headache	76.34	6
7	Necrosis	67.86	133
8	Weight Loss	66.76	37
9	Carcinoma, Renal Cell	59.33	52
10	Bradycardia	57.92	24
11	Disease Models, Animal	43.94	106
12	Hallucinations	39.15	3
13	Abnormalities, Drug-Induced	38.3	5
14	Atrial Fibrillation	31.38	18
15	Urinary Bladder Neoplasms	31.19	102
16	Kidney Diseases	30.75	126
17	Coma	29.82	7
18	Exanthema	28.97	6
19	Long QT Syndrome	28.96	31
20	Tachycardia, Ventricular	27.55	10

Figure 43. Results for APAP search in ctdbase.org. Curated chemical–disease data were retrieved from the Comparative Toxicogenomics Database (CTD), MDI Biological Laboratory, Salisbury Cove, Maine, and NC State University, Raleigh, North Carolina. World Wide Web (URL: http://ctdbase.org/). [June, 2023]

Line of Evidence	Reliability	Relevance	Weight Assigned
In silico	LOW to MEDIUM	LOW	LOW

Reliability is assessed as LOW to MEDIUM because the computational terms are defined and linked through rigorous statistical tools, but they are dependent upon user and study-defined investigations, assays, or publication trends. Relevance is set to LOW as a in silico enrichments or activity calls in high throughput data have several major limitations in modeling human health and disease. Therefore, overall weight is assigned as **LOW**.

XIII. WOE SUMMARY RISK CONCLUSION

Line of Evidence	Reliability	Relevance	Weight Assigned	Signal
In Silico	LOW to MEDIUM	LOW	LOW	Positive
In Vitro/ Ex Utero	HIGH	MEDIUM	MODERATE	Positive
In Vivo	MEDIUM	HIGH	MODERATE to HIGH	Positive

All lines of evidence have positive signals. APAP increases the risk of adverse neurodevelopmental outcomes in humans when used in accordance with the dosing information on the product label.

XIV. CONCLUSIONS

As discussed above, it is my opinion to a reasonable degree of scientific certainty based on my analysis of the weight of the evidence in this case as described above that APAP is a developmental neurotoxicant capable of causing neurodevelopmental disorders in both humans and animals. While epidemiological studies may have confounders that must be controlled for, preclinical animal models are not confounded in the same way because the test subjects can be isolated from confounding variables.

Further, the heterogeneity of the results is not a reason to dismiss the effects of APAP shown in these individual studies. First, the effects of APAP on the body are so complex and multifaceted that no single study design could capture them all. In addition, preclinical evidence of APAP neurodevelopmental toxicity does not require uniformity in design, dose, or hepatic involvement or noninvolvement in order to present relevant data. Though individual studies may show mixed or bidirectional results, neurodevelopmental perturbation of prenatal APAP can manifest in various ways in terms of directionality. In addition, preclinical toxicology data is often inconsistent, even in a regulatory context (Leuchtefeld et al., 2018). Even in straightforward toxicological endpoints under relatively strict regulatory designs, results are not fully consistent across labs. This principle is especially true in realms like developmental neurotoxicology, which is far from straightforward. The heterogeneity of ultimate endpoints seen in the preclinical studies described in this report makes sense given the context of the extremely delicate cascading cellular processes disturbed by APAP use. This heterogeneity is also present in human observational studies, which have shown other neurodevelopmental issues, including hyperkinetic disorders, also associated with APAP use. In brief, preclinical studies show that prenatal APAP perturbs a wide array of cascading neurodevelopmental processes, which are complex enough to cause heterogeneous outcomes depending on inter-subject variability, timing of exposure, and other factors.

[redacted]

[redacted] Most importantly, hepatoxicity can occur even with APAP use at therapeutic doses (Vitols, 2013; Sabaté et al., 2011; Kearns et al., 1998). APAP hepatotoxicity is incompletely understood (Hinson et at., 2010), thus, creating a single delineation between no toxic hepatic involvement and toxic hepatic involvement is naïve under such equivocal understandings, and given that this organ is a primary site of xenobiotic metabolism that stresses the organ under therapeutic and supratherapeutic doses alike.

Internal documents show that the manufacturers of APAP products and their contractors were intensely critical of any toxicology study that failed to show dose-response. Certainly, requiring that there is dose-response is a classic toxicology feature and is more important when there are concerns about causality. However, the stance that studies which did not examine a dose-response relationship should be discounted or discredited is inappropriate for multiple reasons. First, when all other variables are held constant, there are no concerns about the causal relationship between the drug and the outcome in a single dose toxicological study. In addition, numerous high quality preclinical in vivo studies are performed with single concentrations of a chemical (e.g., Church et al., 2018; Cui et al., 2022) demonstrating that a lack of dose-response data is not an indicator of poor-quality research. In other words, the preference towards data showing a dose-response relationship must be viewed in its proper context. Dose-response in preclinical research is less critical when the substance being tested is, like APAP, one that has been used hundreds of times in vivo in rodent studies and has been on the market as an over-the-counter pharmaceutical for nearly a century. Finally, requiring a dose-response relationship to be demonstrated in every single toxicology study ignores the reality that the labs performing these studies, unlike Johnson & Johnson, have relatively limited resources and may not be able to exponentially multiply the cost of their studies in order to examine the effects of multiple doses on individual subjects. Many of the studies criticized for failure to show a dose-response effect were not designed to show such an effect, nor should their results be dismissed for that reason. Instead, those studies should be viewed in context as part of a comprehensive body of evidence showing APAP's potential to act as a developmental neurotoxicants.

Certain preclinical studies may have weaknesses based on the route used for administration of APAP (*e.g.*, criticisms of subcutaneous or peritoneal injection and gavage). Though there is a possibility of those types of administration causing stress in the treated animals, the fact that controls are administered in the same manner undermines that argument. Further, APAP is administered by injection (subcutaneous and intravenous) to people. Additionally, the route of administration can affect the T_{max} and C_{max} APAP (IV administration led to earlier peak concentrations and higher concentrations in brain) but generally does not markedly affect the area under the curve (AUC highly overlapping in ranges) in both plasma and cerebrospinal fluid (see Singla et al., 2012). Though the route of administration should be considered when reviewing the results of a particular study, the fact that APAP was not orally administered does not result in a nullity of the findings.

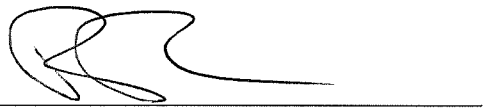
The documents I have reviewed from the manufacturers of APAP appear to attempt to decontextualize the studies, attacking individual research teams in a vacuum for sometimes petty shortcomings including spelling errors. However, it is my opinion that the preclinical research on this topic must be viewed as a whole in order to undergo a rigorous weight of evidence analysis. When viewed in that context, the evidence that APAP harms neurodevelopment is overwhelming.

Despite ample evidence of neurotoxicity in preclinical studies, which stretch back to as early as the 1980s, as well as observational human studies, APAP continues to be sold and recommended to pregnant women with no specific warning regarding its potential effects on neurodevelopment. [redacted]

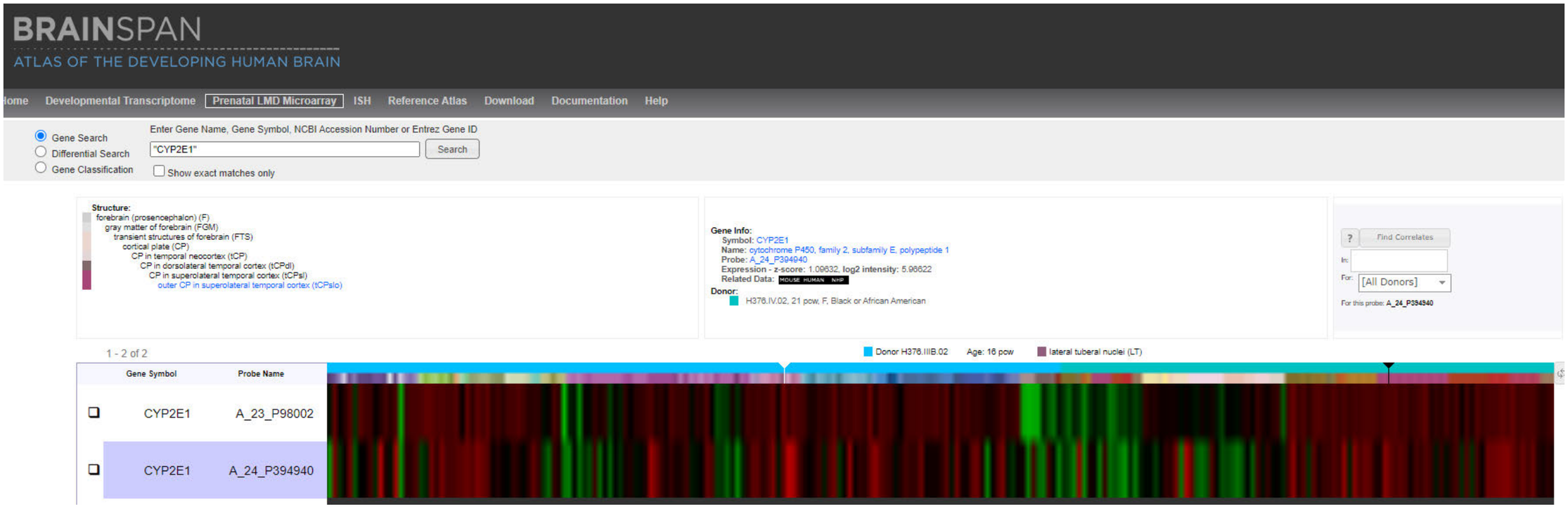
The foregoing opinions are substantively identical to the opinions stated in my June 16, 2023 report. All opinions offered herein are held to a reasonable degree of scientific certainty.

Dated: June 21, 2023

Respectfully submitted,



Brandon L. Pearson, MS, PhD


BRAINSPAN
ATLAS OF THE DEVELOPING HUMAN BRAIN
Developmental Transcriptome
Prenatal LMD Microarray
ISH
Reference Atlas
Download
Documentation
Help
Gene Search
Differential Search
Gene Classification
Enter Gene Name, Gene Symbol, NCBI Accession Number or Entrez Gene ID
"CYP2E1"
Search
Show exact matches only
Structure:
forebrain (prosencephalon) (F)
gray matter of forebrain (FGM)
transient structures of forebrain (FTS)
cortical plate (CP)
CP in temporal neocortex (tCP)
CP in dorsolateral temporal cortex (tCPdl)
CP in superolateral temporal cortex (tCPsl)
outer CP in superolateral temporal cortex (tCPslo)
Gene Info:
Symbol: CYP2E1
Name: cytochrome P450, family 2, subfamily E, polypeptide 1
Probe: A_24_P394940
Expression - z-score: 1.09632, log2 intensity: 5.98622
Related Data: MOUSE HUMAN NHP
Donor:
H376.IV.02, 21 pcw, F, Black or African American
Find Correlates
For: [All Donors]
For this probe: A_24_P394940
1 - 2 of 2
Donor H376.IIIB.02
Age: 16 pcw
lateral tuberal nuclei (LT)
Gene Symbol
Probe Name
CYP2E1
A_23_P98002
CYP2E1
A_24_P394940

Appendix A

PMID	Title	Authors	Citation
36980298	High-Throughput Screening Assay for Detecting Drug-Induced Changes in Synchronized Neuronal Oscillations and Potential Seizure Risk Based on Ca(2+) Fluorescence Measurements in Human Induced Pluripotent Stem Cell (hiPSC)-Derived Neuronal 2D and 3D Cultures	Lu HR, Seo M, Kreir M, Tanaka T, Yamoto R, Altrocchi C, van Ammel K, Tekle F, Pham L, Yao X, Teisman A, Gallacher DJ.	Cells. 2023 Mar 21;12(6):958. doi: 10.3390/cells12060958.
36934829	Fed intestinal solubility limits and distributions applied to the Developability classification system	Silva MI, Khadra I, Pyper K, Halbert GW.	Eur J Pharm Biopharm. 2023 May;186:74-84. doi: 10.1016/j.ejpb.2023.03.005. Epub 2023 Mar 17.
36918128	Functional and targeted proteomics characterization of a human primary endothelial cell model of the blood-brain barrier (BBB) for drug permeability studies	Gomez-Zepeda D, Perrière N, Glacial F, Taghi M, Chhuon C, Scherrmann JM, Sergent P, Moreau A, Denizot C, Parmentier Y, Cisternino S, Decleves X, Menet MC.	Toxicol Appl Pharmacol. 2023 Apr 15;465:116456. doi: 10.1016/j.taap.2023.116456. Epub 2023 Mar 12.
36798113	Entry of the antipsychotic drug, olanzapine, into the developing rat brain in mono- and combination therapies	Huang Y, Qiu F, Habgood M, Nie S, Dziegielewska K, Saunders N.	F1000Res. 2022 Dec 2;11:1417. doi: 10.12688/f1000research.128074.2. eCollection 2022.
36690711	Developmental changes in the extent of drug binding to rat plasma proteins	Qiu F, Dziegielewska KM, Huang Y, Habgood MD, Fitzpatrick G, Saunders NR.	Sci Rep. 2023 Jan 23;13(1):1266. doi: 10.1038/s41598-023-28434-1.
36549432	Sex-specific neurobehavioral and prefrontal cortex gene expression alterations following developmental acetaminophen exposure in mice	Baker BH, Rafikian EE, Hamblin PB, Strait MD, Yang M, Pearson BL.	Neurobiol Dis. 2023 Feb;177:105970. doi: 10.1016/j.nbd.2022.105970. Epub 2022 Dec 19.
36197001	Murine endothelial serine palmitoyltransferase 1 (SPTLC1) is required for vascular development and systemic sphingolipid homeostasis	Kuo A, Checa A, Niaudet C, Jung B, Fu Z, Wheelock CE, Singh SA, Aikawa M, Smith LE, Proia RL, Hla T.	Elife. 2022 Oct 5;11:e78861. doi: 10.7554/eLife.78861.
36100070	Interleukin-1β-induced inflammation and acetaminophen during infancy: Distinct and interactive effects on social-emotional and repetitive behavior in C57BL/6J mice	Harshaw C, Warner AG.	Pharmacol Biochem Behav. 2022 Oct;220:173463. doi: 10.1016/j.pbb.2022.173463. Epub 2022 Sep 11.
36058146	Transplacental transfer of acetaminophen in pregnant rats	Chen JH, Lin IH, Sun CK, Yang L, Hsueh TY, Ting CT, Tsai TH.	Biomed Pharmacother. 2022 Oct;154:113613. doi: 10.1016/j.biopha.2022.113613. Epub 2022 Sep 1.
35966180	Synthesis of calcium carbonate-quince bio-composite for programmed and on-demand drug release of paracetamol at target site: a green chemistry approach	Kulsoom R, Sarfraz M, Afzal A, Farooq M, Adnan S, Ashraf MU, Khan SA.	30:1-24. doi: 10.1007/s00289-022-04400-1. Online ahead of print.
35605926	Structured solubility behaviour in bioequivalent fasted simulated intestinal fluids	Abuhassan Q, Khadra I, Pyper K, Augustijns P, Brouwers J, Halbert GW.	Eur J Pharm Biopharm. 2022 Jul;176:108-121. doi: 10.1016/j.ejpb.2022.05.010. Epub 2022 May 20.
35391823	Paracetamol (Acetaminophen) and its Effect on the Developing Mouse Brain	Philippot G, Hosseini K, Yakub A, Mhajar Y, Hamid M, Buratovic S, Fredriksson R.	Front Toxicol. 2022 Mar 22;4:867748. doi: 10.3389/ftox.2022.867748. eCollection 2022.
34923138	Fasted intestinal solubility limits and distributions applied to the biopharmaceutics and developability classification systems	Abuhassan Q, Khadra I, Pyper K, Augustijns P, Brouwers J, Halbert GW.	Eur J Pharm Biopharm. 2022 Jan;170:160-169. doi: 10.1016/j.ejpb.2021.12.006. Epub 2021 Dec 16.
34802359	Assessment of embryo morphology following perinatal exposure to aspirin, ibuprofen and paracetamol using whole embryo culture system	Moungmaithong S, Leung BW, Sahota DS, Wang CC, Leung TY, Poon LC.	J Matern Fetal Neonatal Med. 2022 Dec;35(25):8786-8793. doi: 10.1080/14767058.2021.2005020. Epub 2021 Nov 22.
34615937	Efflux transporters in rat placenta and developing brain: transcriptomic and functional response to paracetamol	Koehn LM, Huang Y, Habgood MD, Nie S, Chiou SY, Banati RB, Dziegielewska KM, Saunders NR.	Sci Rep. 2021 Oct 6;11(1):19878. doi: 10.1038/s41598-021-99139-6.
34383603	Multi- and Transgenerational Outcomes of an Exposure to a Mixture of Endocrine-Disrupting Chemicals (EDCs) on Puberty and Maternal Behavior in the Female Rat	López-Rodríguez D, Aylwin CF, Delli V, Sevrin E, Campanile M, Martin M, Franssen D, Gérard A, Blacher S, Tirelli E, Noël A, Lomniczi A, Parent AS.	Environ Health Perspect. 2021 Aug;129(8):87003. doi: 10.1289/EHP8795. Epub 2021 Aug 12.

34324979	Carnosine dipeptidase II (CNDP2) protects cells under cysteine insufficiency by hydrolyzing glutathione-related peptides	Kobayashi S, Homma T, Okumura N, Han J, Nagaoka K, Sato H, Konno H, Yamada S, Takao T, Fujii J.	Free Radic Biol Med. 2021 Oct;174:12-27. doi: 10.1016/j.freeradbiomed.2021.07.036. Epub 2021 Jul 27.
34170958	Therapeutic doses of acetaminophen with co-administration of cysteine and mannitol during early development result in long term behavioral changes in laboratory rats	Suda N, Cendejas Hernandez J, Poulton J, Jones JP, Konsoula Z, Smith C, Parker W.	PLoS One. 2021 Jun 25;16(6):e0253543. doi: 10.1371/journal.pone.0253543. eCollection 2021.
33836167	Perinatal exposure to paracetamol: Dose and sex-dependent effects in behaviour and brain's oxidative stress markers in progeny	Rigobello C, Klein RM, Debiasi JD, Ursini LG, Michelin AP, Matsumoto AK, Barbosa DS, Moreira EG.	Behav Brain Res. 2021 Jun 25;408:113294. doi: 10.1016/j.bbr.2021.113294. Epub 2021 Apr 6.
33548366	A moving-boundary model of dissolution from binary drug-excipient granules incorporating microstructure	Moroney KM, Kotamarthy L, Muthancheri I, Ramachandran R, Vynnycky M.	Int J Pharm. 2021 Apr 15;599:120219. doi: 10.1016/j.ijpharm.2021.120219. Epub 2021 Feb 4.
33308393	Effect of paracetamol treatment on maternal care and reproductive outcomes in female rat offspring	Aleixo JF, Pereira MRF, Montagnini BG, Pereira MJD, Forcato S, Moreira EG, Ceravolo GS, Vieira ML, Kiss ACI, Gerardin DCC.	Reprod Fertil Dev. 2020 Dec;32(18):1311-1325. doi: 10.1071/RD20007.
32866265	Applicability of hiPSC-Derived Neuronal Cocultures and Rodent Primary Cortical Cultures for In Vitro Seizure Liability Assessment	Tukker AM, Wijnolts FMJ, de Groot A, Westerink RHS.	Toxicol Sci. 2020 Nov 1;178(1):71-87. doi: 10.1093/toxsci/kfaa136.
32792676	Real time monitoring of oxygen uptake of hepatocytes in a microreactor using optical microsensors	Gehre C, Flechner M, Kammerer S, Küpper JH, Coleman CD, Püschel GP, Uhlig K, Duschl C.	Sci Rep. 2020 Aug 13;10(1):13700. doi: 10.1038/s41598-020-70785-6.
32632539	The EU-ToxRisk method documentation, data processing and chemical testing pipeline for the regulatory use of new approach methods	Krebs A, van Vugt-Lussenburg BMA, Waldmann T, Albrecht W, Boei J, Ter Braak B, Brajnik M, Braunbeck T, Brecklinghaus T, Busquet F, Dinnyes A, Dokler J, Dolde X, Exner TE, Fisher C, Fluri D, Forsby A, Hengstler JG, Holzer AK, Janstova Z, Jennings P, Kisitu J, Kobolak J, Kumar M, Limonciel A, Lundqvist J, Mihalik B, Moritz W, Pallocca G, Ulloa APC, Pastor M, Rovida C, Sarkans U, Schimming JP, Schmidt BZ, Stöber R, Strassfeld T, van de Water B, Wilmes A, van der Burg B, Verfaillie CM, von Hellfeld R, Vrieling H, Vrijenhoek NG, Leist M.	Arch Toxicol. 2020 Jul;94(7):2435-2461. doi: 10.1007/s00204-020-02802-6. Epub 2020 Jul 6.
32375466	PiTMaP: A New Analytical Platform for High-Throughput Direct Metabolome Analysis by Probe Electrospray Ionization/Tandem Mass Spectrometry Using an R Software-Based Data Pipeline	Zaitsu K, Eguchi S, Ohara T, Kondo K, Ishii A, Tsuchihashi H, Kawamata T, Iguchi A.	Anal Chem. 2020 Jun 16;92(12):8514-8522. doi: 10.1021/acs.analchem.0c01271. Epub 2020 May 24.
31926975	Can maternal exposure to paracetamol impair reproductive parameters of male rat offspring?	Pereira MRF, Aleixo JF, Cavalcanti LF, Costa NO, Vieira ML, Ceravolo GS, Moreira EG, Gerardin DCC.	Reprod Toxicol. 2020 Apr;93:68-74. doi: 10.1016/j.reprotox.2019.12.007. Epub 2020 Jan 9.
31638891	Altered activity in the nucleus raphe magnus underlies cortical hyperexcitability and facilitates trigeminal nociception in a rat model of medication overuse headache	Potewiratnanond P, le Grand SM, Srikiatkhachorn A, Supronsinchai W.	BMC Neurosci. 2019 Oct 21;20(1):54. doi: 10.1186/s12868-019-0536-2.
31606411	Acute acetaminophen intoxication induces direct neurotoxicity in rats manifested as astrogliosis and decreased dopaminergic markers in brain areas associated with locomotor regulation	Vigo MB, Pérez MJ, De Fino F, Gómez G, Martínez SA, Bisagno V, Di Carlo MB, Scazziota A, Manautou JE, Ghanem CI.	Biochem Pharmacol. 2019 Dec;170:113662. doi: 10.1016/j.bcp.2019.113662. Epub 2019 Oct 10.
31520316	Integrating Carbon Nanomaterials with Metals for Bio-sensing Applications	Sainio S, Leppänen E, Mynttinen E, Palomäki T, Wester N, Etula J, Isoaho N, Peltola E, Koehne J, Meyyappan M, Koskinen J, Laurila T.	Mol Neurobiol. 2020 Jan;57(1):179-190. doi: 10.1007/s12035-019-01767-7. Epub 2019 Sep 14.
31401214	Ammonia-Induced Brain Edema Requires Macrophage and T Cell Expression of Toll-Like Receptor 9	Manakkat Vijay GK, Hu C, Peng J, Garcia-Martinez I, Hoque R, Verghis RM, Ma Y, Mehal WZ, Shawcross DL, Wen L.	Cell Mol Gastroenterol Hepatol. 2019;8(4):609-623. doi: 10.1016/j.jcmgh.2019.08.002. Epub 2019 Aug 8.
30355303	monotropein and deacetyl asperulosidic acid after oral administration of extracts from Morinda officinalis root in rats	Shen Y, Zhang Q, Wu YB, He YQ, Han T, Zhang JH, Zhao L, Hsu HY, Song HT, Lin B, Xin HL, Qi YP, Zhang QY.	BMC Complement Altern Med. 2018 Oct 24;18(1):288. doi: 10.1186/s12906-018-2351-1.

29893936	Multi-Behavioral Endpoint Testing of an 87-Chemical Compound Library in Freshwater Planarians	Zhang S, Hagstrom D, Hayes P, Graham A, Collins ES.	Toxicol Sci. 2019 Jan 1;167(1):26-44. doi: 10.1093/toxsci/kfy145.
29680280	Pediatric oral formulation of dendrimer-N-acetyl-l-cysteine conjugates for the treatment of neuroinflammation	Yellepeddi VK, Mohammadpour R, Kambhampati SP, Sayre C, Mishra MK, Kannan RM, Ghandehari H.	Int J Pharm. 2018 Jul 10;545(1-2):113-116. doi: 10.1016/j.ijpharm.2018.04.040. Epub 2018 Apr 20.
29385849	Development of paracetamol-caffeine co-crystals to improve compressional, formulation and in vivo performance	Latif S, Abbas N, Hussain A, Arshad MS, Bukhari NI, Afzal H, Riffat S, Ahmad Z.	Drug Dev Ind Pharm. 2018 Jul;44(7):1099-1108. doi: 10.1080/03639045.2018.1435687. Epub 2018 Feb 15.
29133240	Prediction of human CNS pharmacokinetics using a physiologically-based pharmacokinetic modeling approach	Yamamoto Y, Välitalo PA, Wong YC, Huntjens DR, Proost JH, Vermeulen A, Krauwinkel W, Beukers MW, Kokki H, Kokki M, Danhof M, van Hasselt JGC, de Lange ECM.	Eur J Pharm Sci. 2018 Jan 15;112:168-179. doi: 10.1016/j.ejps.2017.11.011. Epub 2017 Nov 11.
28955031	Printing of small molecular medicines from the vapor phase	Shalev O, Raghavan S, Mazzara JM, Senabulya N, Sinko PD, Fleck E, Rockwell C, Simopoulos N, Jones CM, Schwendeman A, Mehta G, Clarke R, Amidon GE, Shtein M.	Nat Commun. 2017 Sep 27;8(1):711. doi: 10.1038/s41467-017-00763-6.
28945963	The Analgesic Acetaminophen and the Antipsychotic Clozapine Can Each Redox-Cycle with Melanin	Temoçin Z, Kim E, Li J, Panzella L, Alfieri ML, Napolitano A, Kelly DL, Bentley WE, Payne GF.	ACS Chem Neurosci. 2017 Dec 20;8(12):2766-2777. doi: 10.1021/acschemneuro.7b00310. Epub 2017 Oct 9.
28933267	Neonatal Plasticity of the Nociceptive System: Mechanisms, Effects, and Treatment of Repetitive Painful Procedures During NICU Admittance	den Hoogen NJV, Patijn J, Tibboel D, Joosten EA.	Curr Pharm Des. 2017;23(38):5902-5910. doi: 10.2174/1381612823666170921130320.
28609318	Biochemical and Pharmacological Characterization of a Mice Model of Complex Regional Pain Syndrome	Das V, Kroin JS, Moric M, Buvanendran A.	Reg Anesth Pain Med. 2017 Jul/Aug;42(4):507-516. doi: 10.1097/AAP.0000000000000622.
28337667	Self-assembled polyelectrolyte complexes films as efficient compression coating layers for controlled-releasing tablets	Li W, Huo M, Sen Chaudhuri A, Yang C, Cao D, Wu Z, Qi X.	J Mater Sci Mater Med. 2017 May;28(5):67. doi: 10.1007/s10856-017-5886-7. Epub 2017 Mar 23.
28315722	Risk mitigation for children exposed to drugs during gestation: A critical role for animal preclinical behavioral testing	Zucker I.	Neurosci Biobehav Rev. 2017 Jun;77:107-121. doi: 10.1016/j.neubiorev.2017.03.005. Epub 2017 Mar 16.
28163096	Paracetamol - Effect of early exposure on neurotransmission, spatial memory and motor performance in rats	Blecharz-Klin K, Piechal A, Jawna-Zboińska K, Pyrzanowska J, Wawer A, Joniec-Maciejak I, Widy-Tyszkiewicz E.	Behav Brain Res. 2017 Apr 14;323:162-171. doi: 10.1016/j.bbr.2017.01.051. Epub 2017 Feb 3.
27903287	High content imaging quantification of multiple in vitro human neurogenesis events after neurotoxin exposure	Wu X, Majumder A, Webb R, Stice SL.	BMC Pharmacol Toxicol. 2016 Dec 1;17(1):62. doi: 10.1186/s40360-016-0107-4.
27864744	A Generic Multi-Compartmental CNS Distribution Model Structure for 9 Drugs Allows Prediction of Human Brain Target Site Concentrations	Hartman R, van den Brink W, Wong YC, Huntjens DR, Proost JH, Vermeulen A, Krauwinkel W, Bakshi S, Aranzana-Climent V, Marchand S, Dahyot-Fizelier C, Couet W, Danhof M, van Hasselt JG, de Lange EC.	Pharm Res. 2017 Feb;34(2):333-351. doi: 10.1007/s11095-016-2065-3. Epub 2016 Nov 18.
27492221	Editor's Highlight: Evaluation of a Microelectrode Array-Based Assay for Neural Network Ontogeny Using Training Set Chemicals	Brown JP, Hall D, Frank CL, Wallace K, Mundy WR, Shafer TJ.	Toxicol Sci. 2016 Nov;154(1):126-139. doi: 10.1093/toxsci/kfw147. Epub 2016 Aug 4.
27530293	Development of microparticles for oral administration of the non-conventional radical scavenger IAC and testing in an inflammatory rat model	Passerini N, Albertini B, Sabatino MD, Corace G, Luppi B, Canistro D, Vivarelli F, Cirillo S, Soleti A, Merizzi G, Paolini M.	Int J Pharm. 2016 Oct 15;512(1):126-136. doi: 10.1016/j.ijpharm.2016.08.024. Epub 2016 Aug 12.
27295086	Is There a Causal Relation between Maternal Acetaminophen Administration and ADHD?	Saad A, Hegde S, Kechichian T, Gamble P, Rahman M, Stutz SJ, Anastasio NC, Alshehri W, Lei J, Mori S, Kajs B, Cunningham KA, Saade G, Burd I, Costantine M.	PLoS One. 2016 Jun 13;11(6):e0157380. doi: 10.1371/journal.pone.0157380. eCollection 2016.

27236639	Development of a permeability-limited model of the human brain and cerebrospinal fluid (CSF) to integrate known physiological and biological knowledge: Estimating time varying CSF drug concentrations and their variability using in vitro data	Gaohua L, Neuhoff S, Johnson TN, Rostami-Hodjegan A, Jamei M.	Drug Metab Pharmacokinet. 2016 Jun;31(3):224-33. doi: 10.1016/j.dmpk.2016.03.005. Epub 2016 Apr 4.
26828476	Acetaminophen Induced Hepatotoxicity in Wistar Rats--A Proteomic Approach	Ilavenil S, Al-Dhabi NA, Srigopalram S, Ock Kim Y, Agastian P, Baru R, Choi KC, Valan Arasu M.	Molecules. 2016 Jan 28;21(2):161. doi: 10.3390/molecules21020161.
26617317	Continuous production of controlled release dosage forms based on hot-melt extruded gum arabic: Formulation development, in vitro characterization and evaluation of potential application fields	Kipping T, Rein H.	Int J Pharm. 2016 Jan 30;497(1-2):36-53. doi: 10.1016/j.ijpharm.2015.11.021. Epub 2015 Nov 23.
26616643	Development and validation of a new dynamic computer-controlled model of the human stomach and small intestine	Guerra A, Denis S, le Goff O, Sicardi V, François O, Yao AF, Garrait G, Manzi AP, Beyssac E, Alric M, Blanquet-Diot S.	Biotechnol Bioeng. 2016 Jun;113(6):1325-35. doi: 10.1002/bit.25890. Epub 2015 Dec 10.
26508095	Therapeutic potential of cyclooxygenase-3 inhibitors in the management of glioblastoma	Oksuz E, Atalar F, Tanırverdi G, Bilir A, Shahzadi A, Yazici Z.	J Neurooncol. 2016 Jan;126(2):271-8. doi: 10.1007/s11060-015-1976-x. Epub 2015 Oct 27.
26403385	Feasability of a new process to produce fast disintegrating pellets as novel multiparticulate dosage form for pediatric use	Hoang Thi TH, Lhafidi S, Carneiro SP, Flament MP.	Int J Pharm. 2015 Dec 30;496(2):842-9. doi: 10.1016/j.ijpharm.2015.09.049. Epub 2015 Sep 25.
26390956	Effect of prenatal and early life paracetamol exposure on the level of neurotransmitters in rats--Focus on the spinal cord	Blecharz-Klin K, Joniec-Maciejak I, Jawna K, Pyrzanowska J, Piechal A, Wawer A, Widy-Tyszkiewicz E.	Int J Dev Neurosci. 2015 Dec;47(Pt B):133-9. doi: 10.1016/j.ijdevneu.2015.09.002 . Epub 2015 Sep 25.
25958352	Levetiracetam synergises with common analgesics in producing antinociception in a mouse model of painful diabetic neuropathy	Micov A, Tomić M, Pecikoza U, Ugrešić N, Stepanović-Petrović R.	Pharmacol Res. 2015 Jul;97:131-42. doi: 10.1016/j.phrs.2015.04.014. Epub 2015 May 6.
25734939	Development of a solvent-free analytical method for paracetamol quantitative determination in Blood Brain Barrier in vitro model	Langlois MH, Vekris A, Bousses C, Mordelet E, Buhannic N, Séguard C, Couraud PO, Weksler BB, Petry KG, Gaudin K.	J Chromatogr B Analyt Technol Biomed Life Sci. 2015 Apr 15;988:20-4. doi: 10.1016/j.jchromb.2015.02.019. Epub 2015 Feb 21.
25640291	Development and characterization of a novel, anatomically relevant rat model of acute postoperative pain	Bree D, Moriarty O, O'Mahony CM, Morris B, Bannerton K, Broom DC, Kelly JP, Roche M, Finn DP.	J Pain. 2015 May;16(5):421-35.e1-6. doi: 10.1016/j.jpain.2015.01.010. Epub 2015 Jan 30.
25607892	Differential gene expression patterns in developing sexually dimorphic rat brain regions exposed to antiandrogenic, estrogenic, or complex endocrine disruptor mixtures: glutamatergic synapses as target	Lichtensteiger W, Bassetti-Gaille C, Faass O, Axelstad M, Boberg J, Christiansen S, Rehrauer H, Georgijevic JK, Hass U, Kortenkamp A, Schlumpf M.	Endocrinology. 2015 Apr;156(4):1477-93. doi: 10.1210/en.2014-1504. Epub 2015 Jan 21.
25347995	Analgesic properties of a peripherally acting and GalR2 receptor-preferring galanin analog in inflammatory, neuropathic, and acute pain models	Metcalf CS, Klein BD, McDougle DR, Zhang L, Smith MD, Bulaj G, White HS.	J Pharmacol Exp Ther. 2015 Jan;352(1):185-93. doi: 10.1124/jpet.114.219063. Epub 2014 Oct 27.
24984750	Hepatic organoids for microfluidic drug screening	Au SH, Chamberlain MD, Mahesh S, Sefton MV, Wheeler AR.	Lab Chip. 2014 Sep 7;14(17):3290-9. doi: 10.1039/c4lc00531g.
24913866	Rapid and sensitive liquid chromatography-tandem mass spectrometry method for determination of protein-free pro-drug treosulfan and its biologically active monoepoxy-transformer in plasma and brain tissue	Romański M, Tężyk A, Zaba C, Główka FK.	Talanta. 2014 Sep;127:123-32. doi: 10.1016/j.talanta.2014.03.067. Epub 2014 Apr 4.
24479765	An LC-MS/MS method for the determination of salidroside and its metabolite p-tyrosol in rat liver tissues	Guo N, Ding W, Wang Y, Hu Z, Wang Z, Wang Y.	Pharm Biol. 2014 May;52(5):637-45. doi: 10.3109/13880209.2013.863946. Epub 2014 Jan 30.
24446829	A physiologically based modeling strategy during preclinical CNS drug development	Ball K, Bouzom F, Scherrmann JM, Walther B, Declèves X.	Mol Pharm. 2014 Mar 3;11(3):836-48. doi: 10.1021/mp400533q. Epub 2014 Jan 29.

23940628	Fatty acid amide hydrolase-dependent generation of antinociceptive drug metabolites acting on TRPV1 in the brain	Barrière DA, Mallet C, Blomgren A, Simonsen C, Daulhac L, Libert F, Chapuy E, Etienne M, Högestätt ED, Zygmunt PM, Eschalier A.	PLoS One. 2013 Aug 5;8(8):e70690. doi: 10.1371/journal.pone.0070690. Print 2013.
23472850	Nerve growth factor exhibits an antioxidant and an autocrine activity in mouse liver that is modulated by buthionine sulfoximine, arsenic, and acetaminophen	Valdovinos-Flores C, Gonsebatt ME.	Free Radic Res. 2013 May;47(5):404-12. doi: 10.3109/10715762.2013.783210. Epub 2013 Apr 8.
23071540	Gait analysis in rats with single joint inflammation: influence of experimental factors	Ängeby Möller K, Kinert S, Størkson R, Berge OG.	PLoS One. 2012;7(10):e46129. doi: 10.1371/journal.pone.0046129. Epub 2012 Oct 5.
22454432	Determination of drug toxicity using 3D spheroids constructed from an immortal human hepatocyte cell line	Fey SJ, Wrzesinski K.	Toxicol Sci. 2012 Jun;127(2):403-11. doi: 10.1093/toxsci/kfs122. Epub 2012 Mar 27.
21824100	Antioxidant therapy: current status and future prospects	Firuzi O, Miri R, Tavakkoli M, Saso L.	Curr Med Chem. 2011;18(25):3871-88. doi: 10.2174/092986711803414368.
21771276	Effects of the analgesic acetaminophen (Paracetamol) and its para-aminophenol metabolite on viability of mouse-cultured cortical neurons	Schultz S, DeSilva M, Gu TT, Qiang M, Whang K.	Basic Clin Pharmacol Toxicol. 2012 Feb;110(2):141-4. doi: 10.1111/j.1742-7843.2011.00767.x. Epub 2011 Aug 20.
21593677	Tumor necrosis factor-α affects blood-brain barrier permeability in acetaminophen-induced acute liver failure	Wang W, Lv S, Zhou Y, Fu J, Li C, Liu P.	Eur J Gastroenterol Hepatol. 2011 Jul;23(7):552-8. doi: 10.1097/MEG.0b013e3283470212.
21170329	Acetaminophen induces apoptosis in rat cortical neurons	Posadas I, Santos P, Blanco A, Muñoz-Fernández M, Ceña V.	PLoS One. 2010 Dec 10;5(12):e15360. doi: 10.1371/journal.pone.0015360.
21046686	Determination and pharmacokinetic study of chiisanogenin in rat plasma by ultra performance liquid chromatography-tandem mass spectrometry	Yang C, Yu K, Song Y, Qin F, Li F.	Phytochem Anal. 2011 May-Jun;22(3):225-9. doi: 10.1002/pca.1269. Epub 2010 Nov 2.
21037491	Acetaminophen inhibits status epilepticus in cultured hippocampal neurons	Deshpande LS, DeLorenzo RJ.	Neuroreport. 2011 Jan 5;22(1):15-8. doi: 10.1097/WNR.0b013e3283413231.
20952763	Ondansetron does not block paracetamol-induced analgesia in a mouse model of fracture pain	Minville V, Fourcade O, Mazoit JX, Girolami JP, Tack I.	Br J Anaesth. 2011 Jan;106(1):112-8. doi: 10.1093/bja/aeq277. Epub 2010 Oct 16.
20862299	TRPV1 in brain is involved in acetaminophen-induced antinociception	Mallet C, Barrière DA, Ermund A, Jönsson BA, Eschalier A, Zygmunt PM, Högestätt ED.	PLoS One. 2010 Sep 17;5(9):e12748. doi: 10.1371/journal.pone.0012748.
20804811	Potential protective effects of quercetin and curcumin on paracetamol-induced histological changes, oxidative stress, impaired liver and kidney functions and haematotoxicity in rat	Yousef MI, Omar SA, El-Guendi MI, Abdelmegid LA.	Food Chem Toxicol. 2010 Nov;48(11):3246-61. doi: 10.1016/j.fct.2010.08.034. Epub 2010 Sep 4.
20188153	Neuroprotective effects vary across nonsteroidal antiinflammatory drugs in a mouse model of developing excitotoxic brain injury	Leroux P, Hennebert C, Catteau J, Legros H, Hennebert O, Laudenbach V, Marret S.	Neuroscience. 2010 May 19;167(3):716-23. doi: 10.1016/j.neuroscience.2010.02.042. Epub 2010 Feb 24.
19609937	A human stem cell-based model for identifying adverse effects of organic and inorganic chemicals on the developing nervous system	Buzanska L, Sypecka J, Nerini-Molteni S, Compagnoni A, Hogberg HT, del Torchio R, Domanska-Janik K, Zimmer J, Coecke S.	Stem Cells. 2009 Oct;27(10):2591-601. doi: 10.1002/stem.179.
19452129	A new minimal-stress freely-moving rat model for preclinical studies on intranasal administration of CNS drugs	Stevens J, Suidgeest E, van der Graaf PH, Danhof M, de Lange EC.	Pharm Res. 2009 Aug;26(8):1911-7. doi: 10.1007/s11095-009-9907-1. Epub 2009 May 19.

19376109	Pharmacological modulation of movement-evoked pain in a rat model of osteoarthritis	Chandran P, Pai M, Blomme EA, Hsieh GC, Decker MW, Honore P.	Eur J Pharmacol. 2009 Jun 24;613(1-3):39-45. doi: 10.1016/j.ejphar.2009.04.009. Epub 2009 Apr 16.
19135806	Pretreatment with alternate day modified fast will permit higher dose and frequency of cancer chemotherapy and better cure rates	Johnson JB, John S, Laub DR.	Med Hypotheses. 2009 Apr;72(4):381-2. doi: 10.1016/j.mehy.2008.07.064. Epub 2009 Jan 9.
19010295	Efficacy of tetrathiomolybdate in a mouse model of multiple sclerosis	Hou G, Abrams GD, Dick R, Brewer GJ.	Transl Res. 2008 Nov;152(5):239-44. doi: 10.1016/j.trsl.2008.09.003. Epub 2008 Oct 11.
18226480	Tablet disintegration and drug dissolution in viscous media: paracetamol IR tablets	Parojcić J, Vasiljević D, Ibrić S, Djurić Z.	Int J Pharm. 2008 May 1;355(1-2):93-9. doi: 10.1016/j.ijpharm.2007.11.058. Epub 2007 Dec 5.
18164319	Non-steroidal anti-inflammatory drugs have potent anti-fibrillogenic and fibril-destabilizing effects for alpha-synuclein fibrils in vitro	Hirohata M, Ono K, Morinaga A, Yamada M.	Neuropharmacology. 2008 Mar;54(3):620-7. doi: 10.1016/j.neuropharm.2007.11.010. Epub 2007 Nov 23.
17665109	Development of drug adsorbates onto soluble inorganic silicate glass surface: example with acetaminophen	Hristova Y, Djambaski P, Samuneva B, Rangelova N, Bogdanova S.	J Mater Sci Mater Med. 2008 Feb;19(2):805-11. doi: 10.1007/s10856-007-3190-7. Epub 2007 Aug 1.
17188851	An investigation into the usefulness of generalized regression neural network analysis in the development of level A in vitro-in vivo correlation	Parojcić J, Ibrić S, Djurić Z, Jovanović M, Corrigan OI.	Eur J Pharm Sci. 2007 Mar;30(3-4):264-72. doi: 10.1016/j.ejps.2006.11.010. Epub 2006 Nov 19.
17157777	The orofacial formalin test in the mouse: a behavioral model for studying physiology and modulation of trigeminal nociception	Luccarini P, Childeric A, Gaydier AM, Voisin D, Dallel R.	J Pain. 2006 Dec;7(12):908-14. doi: 10.1016/j.jpain.2006.04.010.
16890412	Reciprocating dialysis tube method: periodic tapping improved in vitro release/dissolution testing of suppositories	Itoh S, Teraoka N, Matsuda T, Okamoto K, Takagi T, Oo C, Danny Kao H.	Eur J Pharm Biopharm. 2006 Nov;64(3):393-8. doi: 10.1016/j.ejpb.2006.06.004. Epub 2006 Jun 28.
16258901	Circadian pattern of spontaneous behavior in monarthritic rats: a novel global approach to evaluation of chronic pain and treatment effectiveness	Millecamps M, Jourdan D, Leger S, Etienne M, Eschalier A, Ardid D.	Arthritis Rheum. 2005 Nov;52(11):3470-8. doi: 10.1002/art.21403.
16083531	Update on cyclooxygenase inhibitors: has a third COX isoform entered the fray?	Hersh EV, Lally ET, Moore PA.	Curr Med Res Opin. 2005 Aug;21(8):1217-26. doi: 10.1185/030079905X56367.
15659293	Nociceptin/orphanin FQ prevents the antinociceptive action of paracetamol on the rat hot plate test	Sandrini M, Vitale G, Pini LA, Lopetuso G, Romualdi P, Candeletti S.	Eur J Pharmacol. 2005 Jan 10;507(1-3):43-8. doi: 10.1016/j.ejphar.2004.11.030. Epub 2004 Dec 15.
15494188	Pain related behaviour in two models of osteoarthritis in the rat knee	Fernihough J, Gentry C, Malcangio M, Fox A, Rediske J, Pellas T, Kidd B, Bevan S, Winter J.	Pain. 2004 Nov;112(1-2):83-93. doi: 10.1016/j.pain.2004.08.004.
15328377	Transgenic mice with a hypomorphic NADPH-cytochrome P450 reductase gene: effects on development, reproduction, and microsomal cytochrome P450	Wu L, Gu J, Cui H, Zhang QY, Behr M, Fang C, Weng Y, Kluetzman K, Swiatek PJ, Yang W, Kaminsky L, Ding X.	J Pharmacol Exp Ther. 2005 Jan;312(1):35-43. doi: 10.1124/jpet.104.073353. Epub 2004 Aug 24.
15231038	Influence of dissolution media composition on drug release and in-vitro/in-vivo correlation for paracetamol matrix tablets prepared with novel carbomer polymers	Parojcić J, Ethurić Z, Jovanović M, Ibrić S, Jovanović D.	J Pharm Pharmacol. 2004 Jun;56(6):735-41. doi: 10.1211/0022357023583.
15157681	Decrease in non-selective, non-sustained attention induced by a chronic visceral inflammatory state as a new pain evaluation in rats	Millecamps M, Etienne M, Jourdan D, Eschalier A, Ardid D.	Pain. 2004 Jun;109(3):214-224. doi: 10.1016/j.pain.2003.12.028.
15157037	Evaluation of efficacy of the perioperative administration of venlafaxine XR in the prevention of postmastectomy pain syndrome	Reuben SS, Makari-Judson G, Lurie SD.	J Pain Symptom Manage. 2004 Feb;27(2):133-9. doi: 10.1016/j.jpainsymman.2003.06.004.

15139514	A dynamic artificial gastrointestinal system for studying the behavior of orally administered drug dosage forms under various physiological conditions	Blanquet S, Zeijdner E, Beyssac E, Meunier JP, Denis S, Havenaar R, Alric M.	Pharm Res. 2004 Apr;21(4):585-91. doi: 10.1023/b:pham.0000022404.70478.4b.
12952554	Is fever suppression involved in the etiology of autism and neurodevelopmental disorders?	Torres AR.	BMC Pediatr. 2003 Sep 2;3:9. doi: 10.1186/1471-2431-3-9. Epub 2003 Sep 2.
12916927	In vitro evaluation of dissolution behavior for a colon-specific drug delivery system (CODES) in multi-pH media using United States Pharmacopeia apparatus II and III	Li J, Yang L, Ferguson SM, Hudson TJ, Watanabe S, Katsuma M, Fix JA.	AAPS PharmSciTech. 2002;3(4):E33. doi: 10.1208/pt030433.
12106010	Development of an in vitro test battery for the estimation of acute human systemic toxicity: An outline of the EDIT project. Evaluation-guided Development of New In Vitro Test Batteries	Clemedson C, Nordin-Andersson M, Bjerregaard HF, Clausen J, Forsby A, Gustafsson H, Hansson U, Isomaa B, Jørgensen C, Kolman A, Kotova N, Krause G, Kristen U, Kurppa K, Romert L, Scheers E.	Altern Lab Anim. 2002 May-Jun;30(3):313-21. doi: 10.1177/026119290203000309.
11934238	Understanding and predicting drug delivery from hydrophilic matrix tablets using the "sequential layer" model	Siepmann J, Streubel A, Peppas NA.	Pharm Res. 2002 Mar;19(3):306-14. doi: 10.1023/a:1014447102710.
11399797	Spatial glutathione and cysteine distribution and chemical modulation in the early organogenesis-stage rat conceptus in utero	Beck MJ, McLellan C, Lightle RL, Philbert MA, Harris C.	Toxicol Sci. 2001 Jul;62(1):92-102. doi: 10.1093/toxsci/62.1.92.
10332066	Evaluation of colonic absorbability of drugs in dogs using a novel colon-targeted delivery capsule (CTDC)	Ishibashi T, Ikegami K, Kubo H, Kobayashi M, Mizobe M, Yoshino H.	J Control Release. 1999 Jun 2;59(3):361-76. doi: 10.1016/s0168-3659(99)00005-x.
9876598	Critical dissolution tests of oral systems based on statistically designed experiments. III. In vitro/in vivo correlation for multiple-unit capsules of paracetamol based on PLS modeling	Abuzarur-Aloul R, Gjellan K, Sjölund M, Graffner C.	Drug Dev Ind Pharm. 1998 Apr;24(4):371-83. doi: 10.3109/03639049809085633.
9396256	[Two cases of acetaminophen-induced pneumonitis]	Akashi S, Tominaga M, Naitou K, Fujisawa N, Nakahara Y, Hiura K, Hayashi S.	Nihon Kyobu Shikkan Gakkai Zasshi. 1997 Sep;35(9):974-9.
8548862	3,5-Disubstituted analogues of paracetamol. Synthesis, analgesic activity and cytotoxicity	Bessems JG, Gaisser HD, Te Koppele JM, Van Bennekom WP, Commandeur JN, Vermeulen NP.	Chem Biol Interact. 1995 Dec 22;98(3):237-50. doi: 10.1016/0009-2797(95)03649-0.
8719538	Aspirin and acetaminophen reduced both Fos expression in rat lumbar spinal cord and inflammatory signs produced by carrageenin inflammation	Honoré P, Buritova J, Besson JM.	Pain. 1995 Dec;63(3):365-375. doi: 10.1016/0304-3959(95)00065-8.
8581296	Application of intracerebral microdialysis to study regional distribution kinetics of drugs in rat brain	de Lange EC, Bouw MR, Mandema JW, Danhof M, de Boer AG, Breimer DD.	Br J Pharmacol. 1995 Nov;116(5):2538-44. doi: 10.1111/j.1476-5381.1995.tb15107.x.
8065795	Chronic treatments with aspirin or acetaminophen reduce both the development of polyarthritis and Fos-like immunoreactivity in rat lumbar spinal cord	Abbadie C, Besson JM.	Pain. 1994 Apr;57(1):45-54. doi: 10.1016/0304-3959(94)90106-6.
1454388	Intracerebroventricular micro-injections of non-steroidal anti-inflammatory drugs abolish reperfusion hyperalgesia in the rat's tail	Gelgor L, Cartmell S, Mitchell D.	Pain. 1992 Sep;50(3):323-329. doi: 10.1016/0304-3959(92)90038-D.
1682911	Acetaminophen does not induce oxidative stress in isolated rat hepatocytes: its probable antioxidant effect is potentiated by the flavonoid silybin	Garrido A, Arancibia C, Campos R, Valenzuela A.	Pharmacol Toxicol. 1991 Jul;69(1):9-12. doi: 10.1111/j.1600-0773.1991.tb00400.x.
1922684	Attenuation of epileptogenesis by nonsteroidal anti-inflammatory drugs in the rat	Wallenstein MC.	Neuropharmacology. 1991 Jun;30(6):657-63. doi: 10.1016/0028-3908(91)90087-r.
2088466	Effects of eicosanoids on the water and sodium balance of the neonate	Reyes JL, Melendez E.	Pediatr Nephrol. 1990 Nov;4(6):630-4. doi: 10.1007/BF00858640.
1977396	High-pressure liquid chromatographic evaluation of cyclic paracetamol-acetylsalicylate and its active metabolites with results of a comparative pharmacokinetic investigation in the rat	Marzo A, Quadro G, Treffner E, Ripamonti M, Meroni G, Lucarelli C.	Arzneimittelforschung. 1990 Jul;40(7):813-7.

2142085	Estimation of the in vivo effect of cyclooxygenase inhibitors on prostaglandin E2 levels in mouse brain	Ferrari RA, Ward SJ, Zobre CM, Van Liew DK, Perrone MH, Connell MJ, Haubrich DR.	Eur J Pharmacol. 1990 Apr 10;179(1-2):25-34. doi: 10.1016/0014-2999(90)90398-p.
2157085	Acute effects of vitamin B6 and fixed combinations of vitamin B1, B6 and B12 on nociceptive activity evoked in the rat thalamus: dose-response relationship and combinations with morphine and paracetamol	Jurna I, Carlsson KH, Kömen W, Bonke D.	Klin Wochenschr. 1990 Jan 19;68(2):129-35. doi: 10.1007/BF01646861.
2561117	Effects of aspirin and paracetamol on ATPases of human fetal brain: an in vitro study	Sarkar AK, Chakraborti A, Saha UK, Bose SK, Sengupta D.	Indian J Exp Biol. 1989 Sep;27(9):802-4.
2764988	Influence of electrophilic character and glutathione depletion on chemical dysmorphogenesis in cultured rat embryos	Stark KL, Harris C, Juchau MR.	Biochem Pharmacol. 1989 Aug 15;38(16):2685-92. doi: 10.1016/0006-2952(89)90555-8.
2609350	Modulation of the embryotoxicity and cytotoxicity elicited by 7-hydroxy-2-acetylaminofluorene and acetaminophen via deacetylation	Stark KL, Harris C, Juchau MR.	Toxicol Appl Pharmacol. 1989 Mar 1;97(3):548-60. doi: 10.1016/0041-008x(89)90260-3.
4086220	On the non-specific antiinflammatory effects of other-than-antirheumatic drugs. Psychotropic drugs, inflammation and antiinflammatory drugs	Marek J.	Int J Tissue React. 1985;7(6):475-504.
6864528	Saturable accumulation of the anionic herbicide, 2,4-dichlorophenoxyacetic acid (2,4-D), by rabbit choroid plexus: early developmental origin and interaction with salicylates	Kim CS, O'Tuama LA, Mann JD, Roe CR.	J Pharmacol Exp Ther. 1983 Jun;225(3):699-704.
7288620	Levels of acetaminophen and its metabolites in mouse tissues after a toxic dose	Fischer LJ, Green MD, Harman AW.	J Pharmacol Exp Ther. 1981 Nov;219(2):281-6.

CV Prepared June 7, 2023

Brandon Pearson, PhD
Phone [redacted]
Email [redacted]

Academic Appointments/Work Experience

11/2017-Present **Assistant Professor** (Tenure Track)
Department of Environmental Health Sciences
Mailman School of Public Health
and Neurobiology and Behavior PhD Program
Columbia University

8/2007-8/2008 **Behavioral Biologist** (Contractor)
Laboratory of Molecular Pathophysiology
National Institute of Mental Health, U.S. National Institutes of Health
Supervisor: Dr. Husseini Manji

6/2000-7/2005 **Student Intern**
Earth and Environmental Science Division
Los Alamos National Laboratory, U.S. Department of Energy
Supervisor: Dr. Jeanne M. Fair

Education

8/2008-5/2012 **Ph.D.** Behavioral Neuroscience
Department of Psychology, and
Pacific Biosciences Research Center
The University of Hawai‘i at Mānoa, Honolulu, HI
Dissertation: Psycho-social phenotype and brain n-sulfated heparan sulfates in *MeCP2* mutant mice. Advisor- Robert Blanchard, PhD

8/2005-1/2008 **M.S**. in Animal Behavior
Department of Animal Behavior
Bucknell University, Lewisburg, PA USA
Thesis: Behavioral correlates of salivary cortisol in Hamadryas baboons (*Papio h. hamadryas*). Advisor- Peter Judge, PhD

8/2000-5/2005 **B.S.** in Psychology, Biology Minor
University of New Mexico, Albuquerque, NM USA

Training

6/2015-10/2017 **Postdoctoral Researcher**
Translational Biogerontology Lab
German Center for Neurodegenerative Diseases, Bonn, Germany
Advisor- Dan Ehninger, MD

6/2012-6/2015	**Postdoctoral Fellow** Department of Cell Biology and Physiology University of North Carolina at Chapel Hill, Chapel Hill, NC Advisor- Mark Zylka, PhD

Honors and Awards

2019	Calderone Junior Faculty Award, Mailman School of Public Health
2018	Career Development Award, NIEHS Center for Environmental Health in Northern Manhattan
2018	Frankel Visiting Keynote Lecturer, Pediatrics, Johns Hopkins Children's Center
2014	Fellowship Supplement, Cell Biology & Physiology, University of North Carolina
2012	Arts & Sciences Research Award, University of Hawaii
2011	Gartley Research Award, Psychology Department, University of Hawaii
2011	Travel Award: Workshop on the Biology of Prosocial Behavior- Emory University
2010	University Nominee to attend the 2011 Nobel Laureate Meeting- Lindau, Germany
2010	International Behavioral Neuroscience Society Travel Grant- Sardinia, Italy
2008	Kelly Services "Going the Extra Mile" Award
2007	Poster Award, American Society of Primatologists Annual Meeting
2004	Psi Chi Honor Society

Professional and Academic Service

2023-present	Early Career Reviewer, Neurotoxicology and Alcohol Study Section, NIH Center for Scientific Review
2023-present	Technical Advisor, Systematic scoping review and evidence mapping project, Environmental Exposures and ASD, NIEHS
2022-present	Scientific Advisory Committee, Reproductive Medicine Consortium, Uniklinikum, Bonn, Germany
2022-present	Member, Learning Opportunities Action Core, Columbia *FORWARD* Program (Fighting Oppression, Racism, and White Supremacy Through Action, Research, and Discourse).
2022-present	Member, Columbia Plastic Pollution Analysis and Sustainable Solutions (PPASS) Working Group, Columbia Climate School
2022	External Review Panel for the Diamond Pilot Project, Department of Neurology, Dartmouth-Hitchcock Medical Center
2022-present	Co-chair, Columbia University Seminar (USEM) "The Future of Aging Research"
2020-present	Facilitator, Self, Social and Global Awareness Series, Columbia Mailman School of Public Health.
2020-present	Judge, Women in STEM (WiSTEM) ENVISION research proposal competition.
2020-present	Columbia SHARP Scholarship Reviewer (Epigenetics Boot Camp, Microbiome Boot Camp, Google Earth Engine Boot Camp, and Quantitative Genomics Training).
2020-2021	Abstract Judge: Neurotoxicity Specialty Section Toshio Narahashi Poster Award Competition, Society of Toxicology
2019-present	Member, Doctoral Admissions Committee, Department of Environmental Health Sciences, Columbia University
2019-present	Affiliate Member, Columbia Zuckerman Institute
2019-2020	Planning and Development Team Member, Collaboratory Fellows Fund, Columbia Data Science Institute, "Integrating Data Science into Environmental Health Sciences" Funded 2019-2020

2019-2020	400 Years of Inequality event planning committee, Mailman School
2019	NIEHS P30 Cross-Center Meeting Planning Committee
2019-present	Full Faculty Member, Columbia USEM: "The Future of Aging Research"
2018-2019	Instructor for Columbia University SHARP Training Skills for Health and Research Professionals Course: *PI Crash Course: Skills for Future or New Lab Leaders* (Project Management Module)
2018-2022	Member, Doctoral Student Qualifying Exam Committee, Department of Environmental Health Sciences, Columbia University
2018-present	Member, MPH Applicant Review Committee, Department of Environmental Health Sciences, Columbia University
2018	External Grant Reviewer: Scientific Evaluation Panel "Contaminants, ecosystems and health" ANR – Agence Nationale de la Recherche / The French National Research Agency
2017	Visiting Instructor (2 weeks), Advanced Rodent Behavior, Histology, and Microscopy, University of Łódź, Poland.
2016-2017	Instructor, Protein Misfolding and Aggregation in Neurodegenerative Diseases Module, Neuroscience MSc Program, Universität Bonn, Germany
2013-2015	University of North Carolina. Biological & Biomedical Sciences Program. Presentation Evaluator
2012	Local Organizing Committee, International Behavioral Neuroscience Society Annual Meeting, Kona, HI
2011-2012	Education and Training Committee, International Behavioral Neuroscience Society

Professional Organizations and Societies

2022-present	Associate Editor, *Frontiers in Cellular Neuroscience*, Cellular Neuropathology Section
2021-2022	Board Member, Huntington's Disease Society of America, Greater New York City Chapter
2021-2022	Member, American Society of Human Genetics
2020-2023	Editorial Board, *Toxics*
2017-present	Member, Society of Toxicology
2017-2018	Editorial Review Board, *Epigenetic Diagnosis & Therapy*
2009-present	Member, Society for Neuroscience
2013	Member, European Brain and Behaviour Society
2009-2012	Member, International Behavioral Neuroscience Society

Journal Reviewer

Aging and Disease; Antioxidants; Autism Research; Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease; Biomolecules; BMC Biology; Brain and Behavior; Brain Research Bulletin; Genes, Brain and Behavior; Cellular Signalling; Current Psychopharmacology; Environmental Epigenetics; Environmental Health Perspectives; Environment International; Environmental Science and Pollution Research; European Journal of Neuroscience; Frontiers in Cellular Neuroscience; Frontiers in Neuroinformatics; International Journal of Developmental Neuroscience; International Journal of Hygiene and Environmental Health; International Journal of Molecular Sciences; iScience; The Journal of the American Association for Laboratory Animal Science; Journal of Environmental Sciences; Journal of Personalized Medicine; Metabolites; Molecular Autism; Neuroscience Letters; Neurotoxicology and Teratology; Pharmacology, Biochemistry & Behavior; Physiology & Behavior; PLoS Genetics; PLoS One; Research in Autism Spectrum Disorders; Scientific Reports; Translational Psychiatry

Funding

Present Competitive Research Support

1P42ES033719-01 Pearson, Mann (MPI) 9/21/22-6/30/27
COLUMBIA UNIVERSITY AND NORTHERN PLAINS PARTNERSHIP FOR THE SUPERFUND RESEARCH PROGRAM
Sub-Project 5035: Causal molecular mechanisms linking drinking water metal exposures to cardiometabolic disease
Role: PI

Taub Institute, Columbia University Kizil, Pearson (MPI) 9/01/21-8/31/23
COMPARATIVE SINGLE CELL TRANSCRIPTOMICS IN ZEBRAFISH AND KILLIFISH AFTER ACUTE AMYLOID TOXICITY
Role: PI

R01ES032954 Re, Hilpert (MPI) 09/08/21-6/30/26
NEUROTOXIC AND NEURODEGENERATIVE RISKS FROM CHRONIC EXPOSURE TO METAL MIXTURES IN E-CIGARETTE AEROSOL
Role: Co-I

R21 ES032913-01 Pearson (PI) 04/01/21-03/31/23
ENVIRONMENTAL DRIVERS OF TRINUCLEOTIDE REPEAT INSTABILITY AND HUNTINGTON'S DISEASE ONSET
Role: PI

R24 ES029489-01A1 Perera (PI) 07/01/19-06/30/24
MAINTAIN AND ENRICH RESOURCE INFRASTRUCTURE FOR EXISTING ENVIRONMENTAL EPIDEMIOLOGY COHORTS
Role: Co-I

R01ES030950 Margolis (PI) 07/01/19-07/31/24
ENVIRONMENTAL BISPHENOL EXPOSURE, INFANT BRAIN AND BEHAVIOR: HUMAN AND ANIMAL MODELS
Role: Co-I

Past Competitive Research Support

P30 ES009089-20 Baccarelli (PI) 08/01/20-07/31/21
Center for Environmental Health in Northern Manhattan – Pilot Grant
NEURODEVELOPMENTAL AND BEHAVIORAL TOXICITY OF PRENATAL ACETAMINOPHEN EXPOSURE IN MICE
Role: Pilot Award PI

NCN 2017/26/D/ST8/00196 (Nawrotek) 2018-2021
National Science Centre of Poland, SONATA 13 Grant
HYBRID IMPLANTS CONTROLLING DNA DEMETHYLATION FOR THE REGENERATION OF THE PERIPHERAL NERVOUS SYSTEM

Role: mentor (in kind)

Calderone Junior Faculty Prize Pearson (PI) 01/01/19-12/31/19
EXPERIMENTAL APPROACH TO PREVENT THE INTERGENERATIONAL HEALTH IMPACTS OF ADVANCED PATERNAL AGE
Role: PI

P30 ES009089-20 Baccarelli (PI) 05/01/18-04/30/19
Center for Environmental Health in Northern Manhattan Career Development Program
Role: PI

2013-2014
NICHD EXTRAMURAL LOAN REPAYMENT PROGRAM (LRP) FOR PEDIATRIC RESEARCH
Role: PI

2012-2013
NICHD 2T32HD040127-11A1
POSTDOCTORAL RESEARCH IN NEURODEVELOPMENTAL DISORDERS
PI: Joe Piven, MD
Role: Fellow

Educational Contributions

Direct Teaching

2023-present	Faculty Consultant, CAPSTONE Project, International Lead Toxicity Project with Vital Strategies
2019-present	Guest lecturer. *Big Molecular Data* in P8321 Introduction to Data Science for Environmental Health. Columbia University. One lecture.
2019-present	Instructor, P6070 Integration of Science and Practice. Columbia University
2018-present	Instructor, P8334 Computational Toxicology. Columbia University
2018-present	Guest lecturer, P8322. Environmental Determinant of Health II (EDHII) *Environmental factors and neuropsychiatric diseases.* Columbia University. One lecture per year.
2017	Visiting lecturer, *Advanced rodent behavior and immunohistochemistry*. University of Łódź, Poland. (Two weeks, 80 hours)
2015-2017	Instructor, Neuroscience M.Sc. Program, Summer Term Module WPM18 *Protein Misfolding and Aggregation in Neurodegenerative Diseases*, Universität Bonn, Bonn, Germany (3 days: 24 hours per year, Fall term)
2012-2015	Ethics Training Facilitator- *Responsible Conduct of Research* for Biological & Biomedical Science Program (BBSP) MD/PhD students, UNC-Chapel Hill.
2011	Instructor- *Social Neuroscience*, University of Hawaii (Summer term 400-level writing intensive course, 3 credits). This was a new course I designed and offered.
2011	Instructor- *Introduction to Psychobiology*, University of Hawaii (200-level large lecture course, 3 credits)

Advising and Mentorship

Columbia University:

2023-present	Mentor, Claudia Dabie, Program to Inspire Minority Undergraduates in Environmental Health Science Research (PrIMER) Research Student, Fordham University
2023-present	Mentor, Molly Durawa, Barnard College Summer Research Institute Student
2022-present	Co-Advisor (primary advisor), Catherine Lucey, PhD student, Environmental Health Sciences Department
2022-present	Co-Mentor, Farzana Kastury PhD, Fulbright Postdoctoral Fellow, University of South Australia, Currently a Fellow (equivalent to Asst Prof) at University of South Australia
2021-2022	Advisor, Kat Lau, MS thesis, Currently a PhD student at NYU
2020-present	Co-Advisor (primary advisor), Sarah McLarnan, PhD candidate, Columbia University
2020-2021	Advisor, Carine Lama, MS thesis, Currently in medical school at WashU St. Louis.
2020-2021	Advisor, Léa Jean-François, Program to Inspire Minority Undergraduates in Environmental Health Science Research (PrIMER) Research Student, Barnard College, Currently MS in Toxicology Student, Columbia University
2019-2022	Co-Advisor, Brennan Baker, PhD student, Environmental Health Sciences Department, Currently a postdoc at University of Washington
2019-2021	Mentor, Dr. Katarzyna Nawrotek, Visiting Scholar from Lodz University of Technology, Poland, Currently adjunct faculty at Lodz University of Tech,
2019-2020	Advisor, Urvashi Thopte, MA Biotechnology, currently a PhD student at UCSF
2019-2021	Advisor, Joshua Barber, MA Biotechnology, Currently runs all aquatic capabilities at Columbia
2018-2021	Advisor, Stephanie Calluori, Barnard College, Research Thesis and Summer Research Institute, Currently a PhD student at Columbia
2018-2019	Advisor, Sun Young Park, MPH practicum, Currently PhD candidate in Toxicology at NYU
2018-2019	Advisor, Sarah McLarnan, MPH practicum and thesis mentor. Currently PhD student, Columbia
2018-2019	Advisor, Jacintha Halaby, Program to Inspire Minority Undergraduates in Environmental Health Science Research (PrIMER) Research Student, Hunter College (CUNY)
	German Center for Neurodegenerative Diseases:
2017	Supervisor, Elif Toprak, Bachelor Student Summer Research, Ankara University, Turkey
2016	Supervisor, Leonie Posselt, Practicum and Bachelor Thesis: Universität Bonn. Currently a doctoral student at University of Copenhagen. *co-author Pearson et al, 2018 *Epigenomics*
	University of North Carolina:
2015	Supervisor, Sayra Garcia, University of New Mexico, Summer Undergraduate Research Experience (SURE) Program. Currently PhD student, Albert Einstein College of Medicine
2014	Supervisor, Alice Metz, Summer Undergraduate Research Experience (SURE) Program University of Massachusetts- Amherst. Currently a Genetic Counseling Student, Dana Farber Cancer Institute.
2014	Supervisor, Alexander Carlson, Biological & Biomedical Sciences Program (BBSP) MD/PhD rotation student, Currently a pediatrics resident at UCSD.
2013	Supervisor, Reid Olsen, Biological & Biomedical Sciences Program (BBSP) rotation student, Currently a molecular pharmacologist at Exscientia.
2013	Supervisor, Chad Sauvola, Wofford College: Currently a postdoc at MIT
	University of Hawaii:
2011	Supervisor, Jaclyn Bettis, John A. Burns School of Medicine Graduate Rotation Student *co-author Pearson et al, 2012 *Behavioural Brain Research* 233(1), 99-104

2011	Lace Yamomoto, Undergraduate Research Assistant, *co-author Pearson et al, 2012 *Behavior Genetics* 42(2), 299-312
2010	Larry Oasay, Minority Access to Research Careers (MARC) Student, *co-author Pearson et al, 2011 *Genes, Brain and Behavior* 10(2), 228-235

Publications

Peer-Reviewed Research Publications

In Review

Baker BH, Zhang S, Simon JM, McLarnan SM, Chung WK, Pearson BL (Resubmitted). Environmental carcinogens disproportionally mutate genes implicated in neurodevelopmental disorders. https://doi.org/10.1101/2021.12.17.473207 (Preprint). Accompanying web tool: www.environmentalmutation.com

Published

1. Baker BH, Rafikian EE, Hamblin PB, Straight MD, Yang M, **Pearson BL**. (2023). Sex-specific neurobehavioral and prefrontal cortex gene expression alterations following developmental acetaminophen exposure in mice. ***Neurobiology of Disease*** 177: 105970.

2. Xie K, Fuchs H, Scifo E,…**Pearson BL**…Ehninger D (49 authors) (2022). Deep phenotyping and lifetime trajectories reveal limited effects of longevity regulators on the aging process in C57BL/6J mice. ***Nature Communications*** 13, 6830.

3. Nawrotek K, Rudnicka K, Gatkowska J, Michlewska S, **Pearson BL**, Wieczorek M (2021). Ten-eleven translocation methylcytosine dioxygenase 3-loaded microspheres penetrate neurons in vitro causing active demethylation and neurite outgrowth. ***Journal of Tissue Engineering and Regenerative Medicine*** 15(5): 463-474.

4. Kobrzyckna A, Napora P, **Pearson BL**, Pierzchała-Koziec K, Szewczyk R, Wieczorek M (2019). Peripheral and central compensatory mechanisms for impaired vagus nerve function during peripheral immune activation. ***Journal of Neuroinflammation*** 16: 150.

5. **Pearson BL**, Posselt LP, Henzel KS, Ehninger D (2018). Limited efficacy of somatic cell lysis buffer to purify laboratory mouse sperm. ***Epigenomics*** 10(6):689-69.

6. Xie K*, Ryan DP*, **Pearson BL***, Henzel KS, Neff F, Vidal RO, Hennion M, Lehmann I, Schleif M, Schröder S, Adler T, Rathkolb B, Rozman J, Schütz A, Prehn C, Edvar M, Weiergräber M, Adamski J, Busch DH, Ehninger G, Matynia A, Jackson WS, Wolf E, Fuchs H, Gailus-Durner V, Bonn S, Hrabe de Angelis M, Ehninger D. (2018). Epigenetic alterations in longevity regulators, reduced lifespan, and exacerbated aging-related pathology in old father offspring mice. ***Proceedings of the National Academy of Sciences USA*** 115(10):E2348-E2357. *Equal contribution. (F1000 Recommendation)

7. Ryan DP, Henzel KS, **Pearson BL**, Siwek ME, Papazoglou A, Paesler K, Müller R, Xie K, Schröder S, Becker L, Garrett L, Hölter SM, Neff F, Racz I, Rathkolb B, Rozman J, Ehninger G, Klingenspor M, Klopstock T, Wolf E, Wurst W, Zimmer A, Fuch H, Guilus-Durner V, Hrabe de Angelis M, Sidiropoulou

K, M Weiergräber M, Ehninger D (2018). A paternal methyl donor-rich diet altered cognitive and neural functions in offspring mice. ***Molecular Psychiatry*** 23(5): 1345-1355.

8. Xie K, Neff F, Markert A, Rozman J, Aguilar-Pimentel JA, Amarie OV, Becker L, Brommage R, Garrett L, Henzel KS, Hölter SM, Janik D, Lehmann I, Moreth K, **Pearson BL**, Racz I, Rathkolb B, Ryan DP, Schröder S, Treise I, Bekeredjian R, Busch DH, Graw J, Ehninger G, Klingenspor M, Klopstock T, Ollert M, Sandholzer M, Schmidt-Weber C, Weiergräber M, Wolf E, Wurst W, Zimmer A, Gailus-Durner V, Fuchs H, Hrabě de Angelis M, Ehninger D (2017). Every-other-day feeding extends lifespan but fails to delay many symptoms of aging in mice. ***Nature Communications*** 8, 155.

9. **Pearson BL**[*], Simon JM[*], McCoy ES, Salazar G, Fragola G, Zylka MJ (2016). Identification of chemicals that mimic transcriptional changes associated with autism, brain aging and neurodegeneration. ***Nature Communications*** 7:11173. [*]Equal contribution.

10. **Pearson BL**, Defensor EB, Blanchard DC, Blanchard RJ (2015). Applying the ethoexperimental approach to neurodevelopmental syndrome research reveals exaggerated defensive behavior in Mecp2 mutant mice. ***Physiology & Behavior*** 146:98-104.

11. **Pearson BL**, Reeder DM, Judge PG (2015). Crowding increases salivary cortisol but not self-directed behavior in captive baboons. ***American Journal of Primatology*** 77:462-7

12. King IF, Yandava CN, Mabb AM, Hsiao JS, Huang H, **Pearson BL**, Calabrese JM, Starmer J, Parker JS, Magnuson T, Chamberlain SJ, Philpot BD, Zylka MJ. (2013). Topoisomerases facilitate transcription of long genes linked to autism. ***Nature*** 501: 58-62. (F1000 Recommendation)

13. **Pearson BL**[*], Corley MJ[*], Vasconcellos A, Blanchard DC, Blanchard RJ (2013). Heparan sulfate deficiency in autistic postmortem brain tissue from the subventricular zone of the lateral ventricles. ***Behavioural Brain Research*** 243: 138-145. [†] Equal contribution (F1000 Recommendation).

14. **Pearson BL**, Bettis JK, Meyza KZ, Yamamoto LY, Blanchard DC, Blanchard RJ (2012). Absence of social conditioned place preference in BTBR T+tf/J mice: relevance for social motivation testing in rodent models of autism. ***Behavioural Brain Research*** 233(1): 99-104.

15. Pobbe RLH, **Pearson BL**, Defensor EB, Bolivar VJ, Young III WS, Lee HJ, Blanchard DC, Blanchard RJ (2012). Oxytocin receptor knockout mice display deficits in the expression of autism-relevant behaviors. ***Hormones and Behavior*** 61(3): 436-444.

16. Blanchard DC, Defensor EB, Meyza KZ, Pobbe RL, **Pearson BL**, Bolivar VJ, Blanchard RJ (2012). BTBR T+tf/J mice: autism-relevant behaviors and reduced fractone-associated heparan sulfate. ***Neuroscience & Biobehavioral Reviews*** 36(1), 285-296. Addendum in: Neurosci Biobehav Rev. 36(10):2370

17. **Pearson BL**, Defensor EB, Pobbe RLH, Yamamoto L, Bolivar VJ, Blanchard DC, Blanchard RJ (2012). MeCP2 truncation in male mice promotes affiliative social behavior. ***Behavior Genetics*** 42: 299-312.

18. Meyza KZ, Blanchard DC, **Pearson BL**, Pobbe RLH, Blanchard RJ (2012). Fractone-associated heparan sulfates show reduced quantity and altered distributions in BTBR T+tf/J mice, a strong model of autism. ***Behavioural Brain Research*** 228(2): 247-253.

19. Sugawara A†, **Pearson BL**†, Blanchard DC, Ward MA (2012). Mouse females devoid of exposure to males during fetal development exhibit increased maternal behavior. ***Psychoneuroendocrinology*** 37(3): 383-395. †Equal contribution

20. **Pearson BL**, Pobbe RLH, Defensor EB, Oasay L, Bolivar VJ, Blanchard DC, Blanchard RJ (2011). Motor and cognitive stereotypies in the BTBR T+tf/J mouse model of autism. ***Genes, Brain and Behavior*** 10, 228-235.

21. Defensor EB, **Pearson BL**, Pobbe RLH, Bolivar VJ, Blanchard DC, Blanchard RJ (2011). A novel social proximity test suggests patterns of social avoidance and gaze-like aversion in BTBR T+ tf/J mice. ***Behavioural Brain Research*** 217(2), 302-308

22. Pobbe RL, Defensor EB, **Pearson BL**, Bolivar VJ, Blanchard DC, Blanchard RJ (2011). General and social anxiety in the BTBR T+tf/J mouse strain. ***Behavioural Brain Research*** 216(1), 446-451.

23. Pobbe RLH, **Pearson BL**, Defensor EB, Bolivar VB, Blanchard DC, Blanchard RJ (2010). Expression of social behaviors of C57BL/6J versus BTBR inbred mouse strains in the visible burrow system. ***Behavioural Brain Research*** 214(2):443-449.

24. **Pearson BL**, Defensor EB, Blanchard DC, Blanchard RJ (2010). C57Bl/6J mice fail to display preference for social novelty in the three-chamber apparatus. ***Behavioural Brain Research*** 213(2): 189-194.

25. Jezierski T, Goreka-Bruzda A, Walczak M, Swiergiel AH, Chruszczewski MH, **Pearson BL** (2010). Operant conditioning of dogs (Canis familiaris) for identification of humans using scent lineup. ***Animal Science Papers and Reports*** 28(1), 81-93.

26. Malkesman O, Scattoni ML, Parredes D, Tragon T, **Pearson B**, Shaltiel G, Chen G, Crawley JN, Manji HK. (2010). The female urine sniffing test (FUST): a novel approach for assessing hedonic behavior in rodents. ***Biological Psychiatry*** 67(9), 864-871. (F1000 Recommendation).

27. Maeng S, Hunsberger J, ***Pearson B***, Yuan P, Wei Y, McCammon J, Schloesser RJ, Zhou R, Du J, Chen G, McEwen B, Reed JC, & Manji HK (2008). BAG1 plays a critical role in regulating recovery from both manic-like and depression-like behavioral impairments. ***Proceedings of the National Academy of Sciences USA*** 105(25), 8766-8771. (F1000 Recommendation).

28. Shaltiel G, Maeng S, Malkesman O, **Pearson B**, Tragon T, Rogawski M, Gaisor M, Luckenbaugh D, Chen G, & Manji HK (2008). Evidence for the involvement of the kainate receptor subunit GluR6 (GRIK2) in mediating behavioral displays related to behavioral symptoms of mania. ***Molecular Psychiatry*** 13, 858-872.

29. **Pearson BL**, Judge PG, & Reeder DM (2008). Effectiveness of saliva collection and enzyme-immunoassay for the quantification of cortisol in socially-housed baboons. ***American Journal of Primatology*** 70(12), 1145-1151.

Reviews, Chapters, Monographs, Editorials

1. Calluori S, Stark R, **Pearson BL** (2023). Gene-environment interactions in repeat expansion diseases: Mechanisms of environmentally induced repeat instability. ***Biomedicines*** 11(2), 515.

2. Margolis AE, Liu R, Conceição VA, Ramphal B, Pagliaccio D, DeSerisy ML, Koe E, Selmanovic E, Raudales A, Emanet N, Quinn AE, Beebe B, **Pearson BL**; Herbstman JB, Rauh VA, Fifer WP, Fox NA, Champagne FA. Convergent neural correlates of prenatal exposure to air pollution and behavioral phenotypes for psychiatric risk: Potential biological and cognitive pathways. ***Neuroscience and Biobehavioral Reviews*** 137: 104645.

3. **Pearson BL**, Ehninger D (2018). Impact of paternal nutrition on epigenetic patterns. ***Epigenomics*** 10(2):115-117

4. **Pearson BL**, Blanchard DC (2018). Emotion as an Evolutionary Adaptive Pattern: the Roles of Context and Cognition. In *The Nature of Emotion (2nd Ed)*. Davidson, Shackman, Fox & Lapate Eds. Oxford University Press

5. **Pearson BL**, Ehninger D (2017). Environmental chemicals and aging. ***Current Environmental Health Reports*** 4:38-43

6. **Pearson BL**, Crawley JN, Eilam D, Pentkowski NS, Summers CH (2016). Curiosity as an approach to ethoexperimental analysis: behavioral neuroscience as seen by students and colleagues of Bob Blanchard. ***Neuroscience & Biobehavioral Reviews*** 76(B): 415-22.

7. Meyza KZ, Defensor EB, Jensen AL, Corley MJ, **Pearson BL**, Pobbe RL, Bolivar VJ, Blanchard DC, Blanchard RJ (2013). The BTBR T+tf/J mouse model for autism spectrum disorders – in search of biomarkers. ***Behavioural Brain Research*** 251, 25-34.

8. Pobbe RLH, **Pearson BL**, Blanchard DC, Blanchard RJ (2012). Oxytocin receptor and Mecp2 knockout mice exhibit altered expression of autism-related social behaviors. ***Physiology & Behavior*** 107(5): 641-648.

9. **Pearson BL**, Blanchard DC, Blanchard RJ. Ch. 18: Social stress effects on defensive behavior and anxiety. In: Conrad CD (Ed) *Handbook of Stress: Neuropsychological Effects on the Brain*. 2011: Wiley-Blackwell.

10. Fair J, Whitaker S, **Pearson B** (2007). Sources of variation in haematocrit in birds. ***Ibis*** 149, 535-552.

Letters to the Editor

1. Henzel KS, **Pearson BL**, Ehninger DE (2017). Novel evidence for paternal dietary influences on cognitive and neural functions in offspring mice. In Response to Bohacek et al., 2017 Letter to Editor regarding Ryan et al., ***Molecular Psychiatry*** doi:10.1038/mp.2017.162

Seminars/Meetings/Invited oral and poster presentations

Poster Presentation. McLarnan SM, Thopte U, DeSantis K, Lucey K, Pearson BL (2023). Mitochondrial toxicity of polycyclic aromatic hydrocarbons in preimplantation mouse embryos. Society of Toxicology (SOT) Annual Meeting, March 19-23, 2023, Nashville, TN

Poster Presentation. Baker BH, Rafikian EE, Hamblin PB, Strait MD, Yang M, Pearson BL (2023). Sex-specific neurobehavioral and prefrontal cortex gene expression alterations following developmental acetaminophen exposure in mice. Society of Toxicology (SOT) Annual Meeting, March 19-23, 2023, Nashville, TN

Kastury F, Bhattarai P, Nelson N, DeSantis K, Cosacak M, Nicholas S, Hu C, Kizil C, Bostick B, Pearson BL (2023). Detection and speciation of iron particles in killifish brain after exposure to particulate matter and its effects on mechanisms of neurodegeneration. Society of Toxicology (SOT) Annual Meeting, March 19-23, 2023, Nashville, TN

Oral Presentation (Virtual). *Environmental exposures and neurodevelopmental disorders.* New York - Presbyterian Center for Autism and the Developing Brain. November 28, 2022.

Oral Presentation, Guest Lecture (Virtual). *Transcriptomics for environmental neurotoxicant discovery*. UNC-Chapel Systems Biology in Environmental Health Course. November 8, 2022

Poster Presentation. Baker BH, Zhang S, Simon JM, McLarnan SM, Chung WK, Pearson BL (2022). Shared vulnerability of neurological and cancer genes to environmental carcinogens. 11th Conference on Metal Toxicity and Carcinogenesis, Montréal, Québec Canada

Poster Presentation. Kastury F, Nelson N, DeSantis K, Nicholas S, Bostick B, Pearson BL. Spatially-resolved spectroscopic detection of iron (Fe) in the brain of a rapid aging fish exposed to particulate matter. International Particle Toxicology Conference, August 2022

Oral Presentation (Pre-recorded virtual session). *Pesticides increase Huntington's Disease (HD) CAG repeat allele instability*. Invited Session: Novel insights into gene-environment interactions from environmental influences on Mendelian disease phenotypes. American Society of Human Genetics Annual Meeting. Online. October 20, 2021.

Oral Presentation (Virtual). *Developmental Origins of Neurological Disorders and Aging*. Columbia Irving Institute Patient Oriented Research (POR) Colloquium. April 13, 2021.

Poster Presentation (Virtual). Jean-Francois LE, Baker BH, Park S, Stark RC, McLarnan SM, Pearson BL. In vitro evaluation of a candidate pesticide initiator of expanded CAG repeats in Huntington's disease. Society of Toxicology Annual Meeting. Online. March 12-16, 2021.

Oral presentation: *Environmental exposures mutate genes involved in developmental disabilities and aging*. Columbia Robert Butler Aging Center Seminar. New York, NY. November 5, 2020.

Poster Presentation. Baker BH, Pearson BL. 2020. Mutations Link Polycyclic Aromatic Hydrocarbon Exposure to Neurodevelopmental Diseases. Society of Toxicology Annual Meeting. Anaheim, CA March 15-19, 2020 (*Cancelled due to COVID-19*).

Poster Presentation. Baker BH, Park S, Stark RC, McLarnan SM, Pearson BL. 2020. Pyraclostrobin Increases CAG Repeats in Cellular Models of Huntington's Disease. Society of Toxicology Annual Meeting. Anaheim, CA March 15-19, 2020 (*Cancelled due to COVID-19*).

Oral presentation: *The life course impacts of extreme, dynamic environments in short-lived model organisms: a focus on aging.* EMBO/EMBL Symposium: The Organism and Its Environment. Heidelberg, Germany. March 2, 2020.

Poster Presentation. Calluori SD*, Ziska L, Pearson BL. 2020. Extreme Temperature Variation: Effects on Aging and Age-Related Disease in the Rapid Aging African Turquoise Killifish. Clinical Climate Change Conference. Icahn School of Medicine at Mount Sinai. January 24, 2020. *Student Poster Award

Oral presentation: *The influence of preconception factors and prenatal exposures in offspring aging*. Center for Research on Reproduction and Women's Health Seminar. University of Pennsylvania. March 27, 2019.

Poster Presentation. Merwin SJ, McLarnan SM, Halaby J, Stark RC, Pearson BL. 2019. Paternal age modulates neurotoxicity of rotenone *in vitro*. Society of Toxicology Annual Meeting, Baltimore, MD March 12, 2019.

Oral presentation: *Experimental Investigation of Life Course and Pre-conception Factors Contributing to Aging and Neurodegeneration.* Columbia Translational Neuroscience Initiative (CTNI) Seminar, New York, NY. September 29, 2018.

Oral presentation: *Translational research and how it can reveal the impacts of environmental factors on neurocognition.* Evan Frankel Visiting Lecture, Grand Rounds, Pediatrics, Johns Hopkins University Children's Medical Center, Baltimore, MD. May 2, 2018

Oral presentation: *New Omics Strategies in Translational Neurotoxicology*. Annual NIEHS p30 Core Centers Meeting, Davis, California. July 18, 2018

Oral presentation: *Multi- and transgenerational neurodevelopmental effects of environmental toxicants*. December 11, 2017. Department of Environmental Health Sciences, Columbia University Mailman School of Public Health

Oral presentation: *Developmental and intergenerational impact of environmental perturbagens on neurological and neuropsychiatric diseases*. 2 June, 2017. Department of Environmental Health Sciences, Columbia University Mailman School of Public Health.

Oral presentation: *New approaches for identifying intra- and inter-generational contributors to brain disease*. Invited Plenary Lecture 10 April, 2017. University of Lodz, Poland.

Poster Presentation. Simon JM, Pearson BL, McCoy ES, Salazar G, Fragola G, Zylka MJ. 2016. Identification of chemicals that mimic transcriptional changes associated with autism, brain aging and neurodegeneration. American Society of Human Genetics Annual Meeting, Vancouver, BC Canada, October 2016.

Poster Presentation. Pearson B, Simon J, McCoy E, Carlson A, Salazar G, Twomey M, Yi J, Zylka M. 2015. Screening environmental pesticides in neurons with RNA-Seq identifies candidate environmental agents for brain disease. UNC Department of Cell Biology & Physiology Research Day and Perl Memorial Symposium. January 23, 2015.

Poster Presentation. Corley MJ, Pearson BL, Blanchard DC, Blanchard RJ. 2013. Extracellular matrix abnormalities in autistic postmortem brain tissue from the subventricular zone of the lateral ventricles. Society for Neuroscience Annual Meeting, San Diego, CA.

Poster Presentation. Pearson BL, Bridges AS, Philpot BD, Zylka MJ. 2013. Pharmacokinetic analysis of indimitecan, a synthetic topoisomerase inhibitor with potential to rescue the Ube3a deficiency in Angelman Syndrome. European Brain and Behaviour Society Meeting, Munich, Germany, September 6-9, 2013.

Poster Presentation. Corley MJ, Meyza KZ, Pearson BL, Blanchard DC, Blanchard RJ. 2012. Sulfate and sulfate-related abnormalities in the BTBR T+tf/J mouse model of autism. Society for Neuroscience Annual Meeting, New Orleans, LA October 13-17, 2012.

Poster Presentation. Blanchard D, Corley MJ, Defensor EB, Jensen AL, Meyza KZ, Pearson BL, Pobbe RLH, Blanchard RJ. 2012. The BTBR T+tf/J mouse model for autism spectrum disorders- in search of biomarkers. Society for Neuroscience Annual Meeting, New Orleans, LA October 13-17, 2012.

Poster Presentation. Meyza KZ, Vasconcellos AJ, Pobbe RLH, Pearson BL, Blanchard DC, Blanchard RJ. 2012. B6 derived Fmr1 KO mice show modest autistic like phenotype. Society for Neuroscience Annual Meeting, New Orleans, LA October 13-17, 2012.

Poster Presentation. Pearson BL, Corley MJ, Blanchard DC, Blanchard RJ. 2012. Elevated brain heparan sulfates in the neurogenic lateral ventricle wall of MeCP2 mutant mice. International Behavioral Neuroscience Meeting, Kona, HI June 5-10, 2012.

Poster Presentation. Meyza KZ, Pearson BL, Pobbe RLH, Blanchard DC, Blanchard RJ. 2012. Abnormal neuronal activation in response to novelty and social interaction in BTBR T+tf/J mice. International Behavioral Neuroscience Meeting, Kona, HI June 5-10, 2012.

Poster Presentation. Pobbe RLH, Pearson BL, Blanchard DC, Blanchard RJ. 2012. Oxytocin receptor and Mecp2 knockout mouse strains display altered expression of autism-related social behaviors. International Behavioral Neuroscience Meeting, Kona, HI June 5-10, 2012.

Oral presentation: *Social adaptation in the mouse*. Invited Symposium: Aggression, Neuromodulation, and Social Adaptation: Lessons from Multiple Animal Models. International Behavioral Neuroscience Society Meeting, Kona, HI USA, June 5-10, 2012.

Poster Presentation. Pearson BL, Sugawara A, Ward MA, Blanchard DC. 2011. Female mice with no male siblings in utero show enhanced pup care and maternal aggression. Society for Social Neuroscience Meeting, Washington DC, November 10-11, 2011.

Poster Presentation. Pobbe RL, Pearson BL, Defensor EB, Bolivar VJ, Blanchard DC, Young III WS, Blanchard RJ. 2011. Oxtr KO mice display social deficits in semi-natural visible burrow systems. Society for Neuroscience Annual Meeting, Washington D.C. November 12-16, 2011.

Poster Presentation. Meyza KZ, Pearson BL, Bettis JK, O'Hanlon SM, Blanchard DC, Blanchard RJ. 2011. Impaired social conditioned place preference in BTBR mice in a social reinforcement paradigm. Society for Neuroscience Annual Meeting, Washington D.C. November 12-16, 2011.

Poster Presentation. Jensen AL, Defensor EB, Pearson BL, Blanchard DC, Blanchard RJ, Dunn AJ. 2011. Neurochemistry of the BTBR T+tf/J mouse model of autism. Society for Neuroscience Annual Meeting, Washington D.C. November 12-16, 2011.

Poster Presentation. Pearson BL, Meyza KZ, Defensor EB, Pobbe RLH, Bolivar VJ, Blanchard DC, Blanchard RJ. 2011. Enhanced sociability, hyper-defensiveness, and decreased cocaine-induced behavioral reactivity in male mice with mecp2-308 mutation. Society for Neuroscience Annual Meeting, Washington D.C. November 12-16, 2011.

Poster Presentation. Blanchard D, Meyza KZ, Pearson BL, Pobbe RLH, Blanchard RJ. 2011. Abnormal lateral ventricles and subventricular extracellular matrix in BTBR T+tf/J mice. Society for Neuroscience Annual Meeting, Washington D.C. November 12-16, 2011.

Poster Presentation. Sugawara A*, Pearson BL*, Blanchard C, Ward MA. 2011. A novel ART model system for studying the effects of sex differences on mouse behavior reveals that females devoid of exposure to males during fetal development exhibit increased maternal defensiveness. Society for the Study of Reproduction, July 31-Aug 4, 2011, Portland, OR USA. *Authors contributed equally.

Oral presentation: *Enhanced sociability, hyper-defensiveness and decreased cocaine-induced behavioral reactivity in male mice with Mecp2-308 mutation*. Special Symposium: Autism Relevant Behaviors of Mouse Models of ASD, International Behavioral Neuroscience Society, Steamboat Springs, CO USA, May 24-29, 2011.

Poster Presentation. Pearson BL, Defensor EB, Pobbe RLH, Blanchard DC, Blanchard RJ. 2010. C57Bl/6J mice fail to display social novelty preference in a modified three-chamber task. Society for Neuroscience 40th Annual Meeting, November 2010. San Diego, CA.

Poster Presentation. Blanchard D, Pobbe RLH, Defensor EB, Pearson BL, Bolivar VJ, Blanchard RJ. 2010. BTBR T+tf/J mice show deficits in seminatural visible burrow systems. Society for Neuroscience Annual Meeting, November 2010. San Diego, CA.

Poster Presentation. Pobbe RL, Pearson BL, Defensor EB, Oasay L, Blanchard D, Blanchard RJ. 2010. Expression of repetitive and stereotyped behaviors of C57BL/6J versus BTBR inbred mouse strains. Society for Neuroscience Annual Meeting, November 2010. San Diego, CA.

Poster Presentation. Defensor EB, Pobbe RLH, Pearson BL, Bolivar VJ, Blanchard DC, Blanchard RJ. 2010 Social anxiety in BTBR T+tf/J mice. 2010. Society for Neuroscience Annual Meeting, November 2010. San Diego, CA.

Poster Presentation. Blanchard RJ, Defensor EB, Pearson BL, Pobbe RLH, Bolivar VJ, Blanchard DC. 2010. Comparison of BTBR T+tf/J and C57Bl/6J mice in the social proximity test. Society for Neuroscience Annual Meeting, November 2010. San Diego, CA.

Poster Presentation. Blanchard DC, Bolivar V, Pobbe R, Defensor E, Pearson BL, Blanchard RJ. 2010. BTBR T+tf/J mice show changes in social behaviors and anxiety, and enhanced stereotypy. Brain Research Meeting: The Emerging Neuroscience of Autism Spectrum Disorders, November 2010, San Diego, CA.

Oral presentation: *Characterization of three-chamber social-novelty seeking in C57Bl/6J mice*. International Behavioral Neuroscience Society, Villasimus, Sardinia, Italy, June 8-13, 2010.

Poster Presentation. Blanchard DC, Pobbe R, Defensor E, Pearson BL, Blanchard RJ. 2010. BTBR T+tf/J mice show social deficits in seminatural visible burrow systems. International Behavioral Neuroscience Society (IBNS) Annual Meeting, Villasimus, Sardinia, Italy, June 8-13, 2010.

Poster Presentation. Blanchard DC, Pobbe R, Defensor E, Pearson BL, Blanchard RJ. 2010. BTBR T+tf/J mice show social deficits in seminatural visible burrow systems. International Behavioral and Neural Genetics Society Meeting Halifax, Nova Scotia, Canada, May 12-16, 2010.